University of Alberta

Serglycin Refines the Granzyme B Endocytosis Pathway During Granule-Mediated Killing

by

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of the

requirements for the degree of Doctor of Philosophy

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Abstract

Cytotoxic T lymphocytes and natural killer cells can eliminate target cells via the directed exocytosis of cytotoxic molecules such as granzymes and perforin. It is believed that to induce apoptosis, granzymes enter the target cell by an endocytic route, therefore granzyme B endocytosis was explored, focusing on two specific aspects. The first hypothesis was that granzyme B endocytosis required dynamin, a critical factor in clathrin-mediated endocytosis. However, dominant-negative dynamin overexpression in target cells partially inhibited granzyme B uptake or granzyme B and perforin-induced apoptosis, suggesting only a partial role for dynamin in granzyme B uptake. The second hypothesis proposed that granzyme B uptake was mediated by the cation-independent mannose 6-phosphate receptor. Soluble mannose 6-phosphate blocked killing by purified granzyme B and adenovirus, but had a negligible effect on granzyme B and perforin-mediated killing, suggesting a mannose 6-phosphate receptor was not critical for granzyme B uptake. Notably, granzyme B is secreted from killer cells bound to the proteoglycan serglycin. Therefore the two hypotheses were re-tested using cytotoxic T lymphocyte degranulate material, which contains the granzyme B-serglycin complex. Both dominant-negative dynamin and mannose 6-phosphate effectively blocked apoptosis induced by degranulate, or degranulate and streptolysin O, respectively. This suggested that when cytotoxic molecules are complexed to serglycin, their interactions with the surface of target cells are refined toward a pathway involving dynamin and a mannose 6-phosphate receptor. In light of this new model, the interactions of serglycin with granzymes A and B were characterized. Granzymes and serglycin were co-precipitated by a biotinylated, soluble cation-independent mannose 6-phosphate receptor,

suggesting the receptor could bind intact serglycin-granzyme complexes. However, serglycin-complexed granzymes A and B were not co-immunoprecipitated, suggesting these granzymes are segregated to distinct serglycin complexes. This disallowed the model that a single receptor might bind a mixed serglycin complex, and thereby co-internalize granzymes A and B. Most importantly, fractionation of degranulate suggested that secreted granzymes are predominantly serglycin-bound, thus it is in this form that the granzymes encounter the target cell. Together, the data imply that under physiological conditions, granzyme B-serglycin will be internalized by the cation-independent mannose 6-phosphate receptor.

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List of Abbreviations

7-AAD	7-amino-actinomycin D
ATCC	American Type Culture Collection
AdV	adenovirus
BAADT ester	butoxylcarbonyl-Ala-Ala-Asp-thiobenzyl ester
bCI-MPR	soluble, biotinylated CI-MPR
BLT ester	N^{α}-benzyloxycarbonyl-L-Lys-thiobenzyl ester
BSA	bovine serum albumin
CD-MPR	cation-dependent mannose 6-phosphate receptor
CD-MPR^{+}	CD-MPR-overexpressing
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CI-MPR	cation-independent mannose 6-phosphate receptor
CI-MPR^{-}	CI-MPR-deficient
CI-MPR^{+}	CD-MPR-overexpressing
CMA	concanamycin A
ConA	concanavalin A
CS-A	chondroitin sulfate A
CTL	cytotoxic T lymphocytes
$\Delta\Psi_m$	mitochondrial inner membrane potential
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DN	dominant negative
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
ECL	enzyme-linked chemiluminexcence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
E/T ratio	effector/target ratio
Fas-L	Fas-ligand

FBS	fetal bovine serum
G6P	glucose 6-phosphate
GAG	glycosaminoglycan
grA	granzyme A
grB	granzyme B
grB-A488	Alexa Fluor 488-conjugated grB
hCTL	human CTL
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HighDegran	high molecular weight (> 100 kDa) degranulate material
HS	heparin or heparan sulfate
Hsp70	heat shock protein 70
IAP	inhibitor of apoptosis protein
IEPD-pNA	acetyl-Ile-Glu-Pro-Asp-paranitroanilide
M6P	mannose 6-phosphate
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatability complex
mMCP	mouse mast cell proteases
MPR	mannose 6-phosphate receptor
nαFas	neutralizing anti-Fas mAb
NK	natural killer
PBS	phosphate-buffered saline
PI	propidium iodide
PLO	pneumolysin O
pfn	perforin
PVDF	polyvinylidene fluoride
RDI	Research Diagnostics Inc.
RHFM	RPMI 1640 medium supplemented with 10% FBS, 25 mM HEPES, 1 mM sodium pyruvated, 100 μM 2-mercaptoethanol, and 100 U/mL each of penicillin and streptomycin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel eletrophoresis

SG	serglycin
SLO	streptolysin O
TBE	Tris/borate/EDTA buffer
TBS	Tris-buffered saline
TCR	T-cell receptor
tet	tetracycline
Tfn-FITC	fluoresein-conjugated transferrin
TMRE	tetramethyl-rhodamine, ethyl ester, perchlorate
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
WT	wild type

Chapter 1

Introduction

1.1. Immune Cells and Granule-Mediated Killing

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are both immune cells with cytotoxic activity that is directed at cellular targets (Janeway et al., 2005). These cytotoxic immune cells maintain the integrity of organs and the whole organism by detecting and eliminating virus-infected and tumorigenic cells. Importantly, CTL and NK cytotoxicity have also been implicated in several pathological events: transplant rejection, graft versus host disease, and autoimmune disease. One of the major mechanisms by which CTL and NK mediate cytotoxicity is through the release of membrane-bounded cytoplasmic granules. These granules contain a variety of cytotoxic effectors that induce apoptosis in the targeted cell. Because of their cytotoxic effects, the granule proteins have been implicated in many of the immune functions of the CTL and NK cells (Barry and Bleackley, 2002; Russell and Ley, 2002; Trapani and Smyth, 2002). Apart from granule-mediated killing, CTL and NK cells may induce death by engaging the death receptor Fas on the surface of the target cell (see **section 1.2.3.**).

1.1.1. Basic Immunobiology of CTL and NK cells

Both CTL and NK cells belong to the branch of the immune system that is responsible for cell-mediated immunity. These cells are distinct in how they recognize target cells: CTL respond to specific antigen, while NK cells are antigen-independent.

When CTL scan potential targets for "non-self" antigens, they detect immunogenic peptides nestled in the cleft of major histocompatability complex (MHC) class I molecules displayed on the surface of cells (figure 1-1a; Janeway et al. 2005).

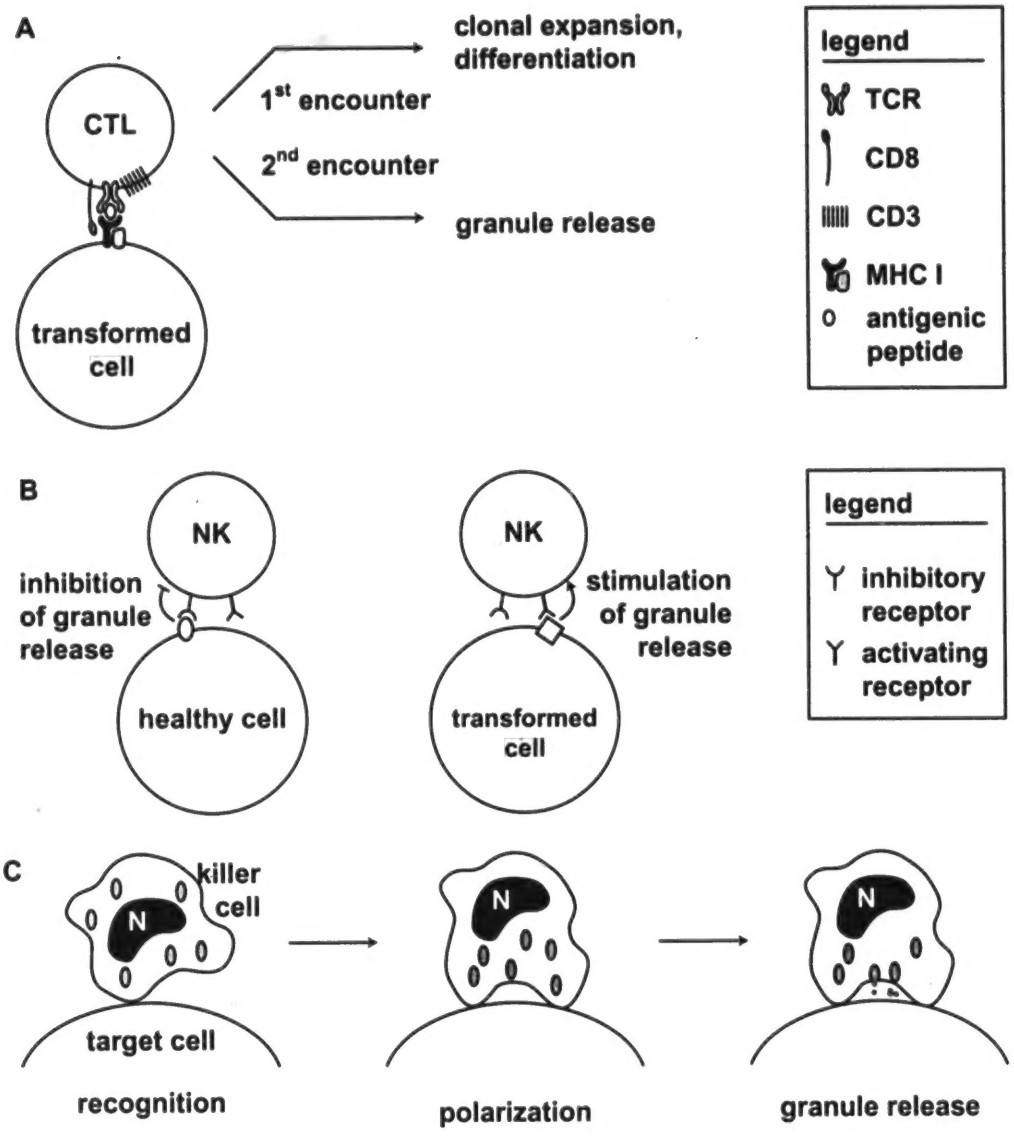

Figure 1-1. Stimulation of granule release from cytotoxic T lymphocytes and natural killer cells.

A, CTL stimulation by a transformed cell (Janeway et al., 2005). B, NK cell signaling induced by healthy and transformed cells (Moretta et al., 2004). C, Granule release is preceded by target cell recognition and polarization of cytotoxic granules. CTL, cytotoxic T lymphcyte; MHC, major histocompatability complex; N, nucleus; NK, natural killer; TCR, T-cell receptor.

CTL recognize this structure through the combined action of an adaptive T-cell receptor (TCR) and the co-receptor CD8. Specific recognition of antigen induces signaling in the CTL through the TCR-associated signaling complex, CD3. The signaling event triggers either of two events, depending on the maturity of the CTL. In a naïve CTL, signaling triggers differentiation of the cell, specifically turning-on genes that encode cytotoxic granule molecules. This priming event also triggers rapid growth, causing expansion of the antigen-specific CTL clone. Following these changes, a CTL is now activated, and subsequent recognition of the specific antigen induces secretion of the cytotoxic granules, which act on the offending cell.

A key distinguishing factor of NK cells is that they express neither the TCR nor the CD3 signaling complexes (Moretta et al., 2004; Janeway et al., 2005; Smyth et al., 2005). Rather, NK cell activity is regulated through a combination of activating and inhibiting receptors (figure 1-1b). As an example of inhibition, NK cells express inhibitory receptors that specifically recognize MHC class I molecules on the surface of potential target cells. Detection of MHC class I on a healthy cell inhibits NK cell activation. In contrast, many virus-infected or tumorigenic cells down-regulate surface MHC class I, thus de-repressing the activity of NK cells. Notably, NK cells also require an activation signal. Cells may induce NK activation by displaying stress markers on their surface. When an appropriate balance of inhibitory and activating signals is received by the NK cell, then, like the CTL, cytotoxic granules are released.

An important difference between CTL and NK cells is that the latter do not require a priming step, and are always armed with cytotoxic granules. Thus, NK cells

can manage a virus infection in early stages, while CTL only mount an effective response after activation and expansion.

1.1.2. The Granule-Release Cytotoxicity Model

When NK cells and activated T cells identify an appropriate target cell, they may eliminate that cell through the release of cytotoxic granules. Within the cytoplasm of the immune cells are dense, membrane-bounded granules. These granules are loaded with a combination of cytotoxic granule-specific molecules, as well as lysosomal proteins. Among the granule-specific molecules are a family of serine proteases called granzymes, a pore-forming molecule perforin (pfn), and the proteoglycan serglycin (SG; Burkhardt et al., 1990; Peters et al., 1991). The lysosomal proteins include cathepsins and lamp-1 (Burkhardt et al., 1990; Peters et al., 1991). The similarity of these cytotoxic granules to lysosomes is further extended to the pH, which is approximately pH 5.4-.5.5 (Burkhardt et al., 1990; Masson et al., 1990). For these reasons, the cytotoxic granules are often described as secretory lysosomes.

The release of the cytotoxic granules is preceded by a specific set of events (figure 1-1c). Firstly, the killer cell engages the target cell to form a tight synapse, such that close contact is established along an extended surface of the plasma membranes. Next, the cytoplasmic granules in the killer cell polarize toward the synapse, and specifically release the granule contents into the synaptic space between the killer and the specific target (Geiger et al., 1982; Kupfer and Dennert, 1984; Yannelli et al., 1986; Peters et al., 1989). This degranulation event is dependent on extracellular calcium influx

(Velotti et al., 1987). While the secreted cytotoxic factors trigger a cell death response in the target cell, the killer cell moves on to identify and eliminate a new target (Zagury et al., 1975; Sanderson, 1976; Rothstein et al., 1978).

1.2. The Death Response in Target Cells is Apoptotic

The cell death that CTL and NK cells induce in target cells has been morphologically characterized as apoptotic. Indeed, this apoptosis can be effected by the release of cytotoxic granules. In particular, the apoptotic activities have been extensively studied for granule proteins granzyme A (grA) and granzyme B (grB), both of which are co-dependent on pfn (Shi et al., 1992a). Notably, the killer cells have a second important mechanism to induce apoptosis in target cells. This alternate pathway requires Fas-ligand (Fas-L) expression on the surface of the killer cell to engage the death receptor Fas on the surface of the target cell (Suda et al., 1993). Though this thesis will focus primarily on granule-mediated killing, distinguishing Fas- from granzyme-mediated pathways is important to the study of CTL- and NK cell-induced apoptotic mechanisms.

1.2.1. Cell Death: Comparing and Contrasting Apoptosis and Necrosis

Apoptotic cell death (reviewed by Jiang and Wang (2004)) has a number of characteristic physical attributes, such as maintained organelle integrity, cell shrinkage, membrane blebbing, chromatin condensation, nuclear membrane disintegration and release of small vesicles called apoptotic bodies from the dying cell. Finally, within the environment of an organ, the dying cell is phagocytosed by neighboring cells.

Importantly, this helps contain the dying process, and thus prevents an inflammatory response. Biochemical events that are detectable markers of apoptosis include externalization of phosphatidyl serine molecules that are normally found on the inner leaflet of the plasma membrane, loss of mitochondrial polarity, condensation of chromatin and/or DNA fragmentation to 200 bp oligomers. In late stages of apoptosis, loss of membrane integrity has also been observed, however this may be an *in vitro* artifact, due to secondary activation of the necrotic pathway (Nicotera and Melino, 2004).

Necrosis is another form of cell death, and may display some of the biochemical attributes of apoptosis, but morphologically is distinct (reviewed by Assunção Guimarães and Linden (2004); and Nicotera and Melino (2004)). Cells swell, organelles are damaged, and the plasma membrane suffers disruption, which leads to the loss of cellular contents into the intercellular space. This cellular debris triggers an inflammatory response, causing swelling and influx of immune cells, therefore setting an important distinction from apoptosis.

1.2.2. Granzyme-Mediated Apoptosis

The effectors of granule-mediated killing that have been best characterized are granzymes A and B, though both are co-dependent on pfn in order to mediate their cytotoxic effects (reviewed by Lieberman (2003); Lord et al. (2003); Roberts et al. (2003); and Trapani and Sutton (2003)). Granules acquire grA and grB through directed targeting, a process that requires a mannose 6-phosphate (M6P) glycosylation tag to mediate efficient trafficking (Burkhardt et al., 1989; Griffiths and Isaaz, 1993). Though

pfn is also targeted to granules, its targeting is via an unknown route, independent of the M6P pathway (Burkhardt et al., 1989).

When either grA or grB are co-delivered with pfn to a target cell, a variety of apoptotic markers are induced: chromatin condensation, DNA damage, and mitochondrial depolarization (Lieberman, 2003; Pardo et al., 2004). Notably, the induction of these markers by grA and grB is distinct both in mechanism and kinetics. Since pathways have been deciphered for grA and grB-induced DNA damage, this marker will be used to describe what is known about some of the mechanistic differences between granzymes A and B.

1.2.2.1. Mechanisms of granzyme A-induced apoptosis

GrA is a serine protease with trypsin-like enzymatic specificity, preferring to cleave substrates after basic residues (Pasternack and Eisen, 1985; Kramer et al., 1986; Masson et al., 1986; Young et al., 1986b). Characterized pathways leading to target cell death involve cleavage of cytoplasmic substrates and, therefore, require grA to enter the cell, which is facilitated by pfn. A grA-dependent pathway leading to DNA damage has recently been characterized (figure 1-2a). GrA induces single-stranded nicks in the DNA of targeted cells (Beresford et al., 1999). It does so by activating a DNase, NM23-H1 (Fan et al., 2003), by cleaving and inactivating the protein SET (Beresford et al., 2001), an inhibitor of the DNase. This allows the translocation of NM23-H1 and cleaved SET from the endoplasmic reticulum to the nucleus, and releases the DNA nicking activity of NM23-H1.

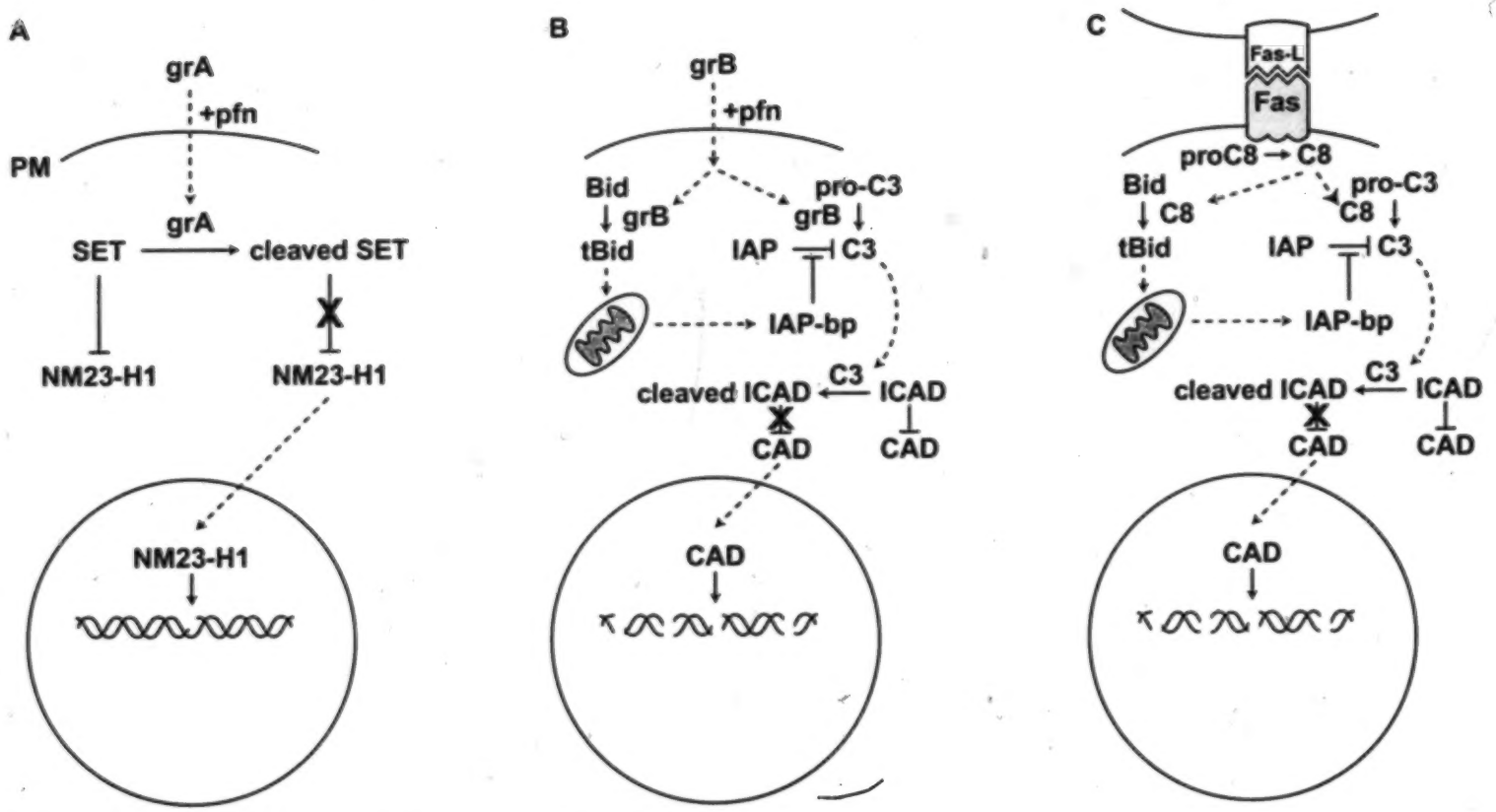

Figure 1-2. Killer cell-induced DNA damage pathways.
Mechanisms of DNA damage induced by grA (A), grB (B), or Fas-L (C). GrA and grB mediate their effects by entering the target cell cytoplasm in a pfn-dependent manner; Fas-L engages the Fas receptor at the cell surface to activate caspase 8 and downstream events. C3/8, caspase 3/8; CAD, caspase-activated DNase; Fas-L, Fas-ligand; grA, granzyme A; grB, granzyme B; IAP, inhibitor of apoptosis protein; IAP-bp, IAP binding protein; ICAD, inhibitor of CAD; pfn, perforin; PM, plasma membrane; tBid, truncated Bid.

1.2.2.2. Mechanisms of granzyme B-induced apoptosis

GrB, like grA, is a serine protease, but it possesses a distinct enzymatic specificity, preferring to cleave substrates after aspartate residues (Murphy et al., 1988; Odake et al., 1991; Poe et al., 1991). In a manner similar to grA, characterized pathways leading to apoptosis require pfn-assisted internalization of grB to induce target cell death. The grB-mediated pathway leading to DNA damage is somewhat more complex than that of grA, and the phenotype is also distinct in that the activated DNase generates 200 bp fragments (figure 1-2b). The pathway is initiated by preferential cleavage of two key substrates, caspase 3 (Darmon et al., 1995; Quan et al., 1996) and bid (Barry et al., 2000; Heibein et al., 2000; Sutton et al., 2000). Caspase 3 is a cysteine protease preferring to cleave substrates after aspartate residues, as grB does. Cleavage of caspase 3 is activating, though the enzymatic activity remains checked by inhibitor of apoptosis proteins (IAPs). The liberation of caspase 3 activity is achieved through the aid of granzyme B-cleaved bid. The truncated bid translocates to the mitochondria, and induces the subsequent mitochondrial release of mitochondrial pro-apoptotic factors, including IAP-binding proteins that antogonize IAPs (Goping et al., 2003; Sutton et al., 2003). The free caspase 3 then goes on to activate the caspase-activated DNase by cleaving and inactivating its inhibitor (Sakahira et al., 1998), in much the same way as grA activates its DNase.

1.2.2.3. The role of perforin in granzyme-mediated apoptosis

A discussion of granzyme-mediated killing must also include pfn, since either granzyme is co-dependent on pfn in order to induce apoptosis of target cells (Shi et al., 1992a; Shi et al., 1992b). Pfn is best known for its calcium-dependent lytic activity

(Masson and Tschopp, 1985; Podack et al., 1985; Liu et al., 1986). Nonetheless, while this activity is sufficient to rupture vesicles and erythrocytes, it is inefficient in causing the death of nucleated cells (Shiver and Henkart, 1991). In contrast, co-delivery of pfn with grB and/or grA leads not only to effective target cell death, but in particular, to apoptotic death, characteristic of killer cell-mediated death (Heusel et al., 1994; Nakajima et al., 1995). This co-dependence of pfn and granzymes led to the proposal of the pfn pore translocation model. This model proposes that pfn forms a pore in the plasma membrane that mediates translocation of granzymes from the extracellular milieu into the cytoplasm of the target cell. The entry of the granzymes into the target cell then leads to the proteolytic activation of apoptotic pathways. However, significant data exist to suggest an alternative granzyme uptake model. Rather, it seems likely that granzymes are first endocytosed, and the role of pfn is to assist the release of granzymes from the endosome into the cytoplasm. Further details will be discussed in **section 1.4.1.**

1.2.3. Fas-Mediated Apoptosis

CTL and NK cells not only kill targets by cytotoxic granules, but also by engaging and activating the death receptor Fas/CD95 on the surface of target cells. Signaling through the Fas receptor is dependent on the expression of Fas-L on the surface of the killer cell (figure 1-2c; reviewed by Linkermann et al. (2003)). Activation of the Fas receptor leads to cleavage and activation of caspase 8, which can cleave caspase 3 and bid in a manner analogous to grB (reviewed by Thorburn (2004)). In continued similarity to grB-induced events, functional activates the caspase-activated DNase to induce double-stranded DNA breaks.

The Fas pathway was distinguished based on calcium independence, which contrasted calcium-dependent granule-mediated killing (Rouvier et al., 1993). It is indeed true that neither Fas/Fas-L conjugation nor the downstream pathways require calcium. However, Fas-L localizes to the granules of the killer cells and is externalized to the surface upon calcium-dependent release of the granules, thus the pathway is regulated upstream by calcium (Bossi and Griffiths, 1999).

1.2.4. Monitoring Apoptosis

To study cell death, two key events are typically tracked: plasma membrane permeabilization and DNA fragmentation (Shi et al., 2000). The first is detected by release of cytosolic factors that have been metabolically labeled with [^{51}Cr]sodium chromate. Chromium released into the medium can be quantified as a measure of overall cell death in response to a death stimulus. To measure DNA fragmentation, cells are first metabolically labeled with either [^{3}H]thymidine, or with [^{125}I]iododeoxyuridine. Then after the desired treatment period with stimulus, plasma membranes are disrupted with Triton X-100, and the cell ghosts, including large nuclear fragments, are isolated by centrifugation. In contrast, small nuclear fragments remain in the supernatant, and are quantified as a measure of cell death.

Both DNA fragmentation and chromium release occur during CTL- and NK cell-induced apoptosis. Therefore these assays are important tools in the study of killer cell-mediated death mechanisms, particularly if applied in combination. However, these

methods are also limited by overlapping events of grA-, grB-, pfn- and Fas-dependent pathways.

1.2.4.1. Distinguishing granzyme A, granzyme B and perforin-dependent events

DNA fragmentation and chromium release during granule-mediated killing are induced by the combined activities of grA, grB, pfn, as well as other granule factors. An important tool in deciphering the relative contributions of these factors was a set of knockout mice in which grA, grB, pfn, or both grA and grB were deleted. From these various knockout mice, splenocytes were isolated to use as cytotoxic effectors in killing assays. Firstly when both grA and grB were deleted, this was insufficient to block killer cell-induced chromium release (Simon et al., 1997; Pardo et al., 2002), though absence of pfn effectively achieved complete inhibition (Simon et al., 1997). Thus pfn must be required to induce chromium release, though it is likely not mediating this effect alone since pfn alone is inefficient in causing cell lysis (Shiver and Henkart, 1991). While it had indeed been shown that combined delivery of grA and pfn, or of grB and pfn is sufficient to induce chromium release (Shi et al., 1992a; Shi et al., 1992b), the studies with the knockout mice suggested that the absence of grA and grB can be compensated by other granule factors. Overall, this suggested that pfn is required for killer cell-mediated cytotoxicity, most likely by cooperating with other granule factors, which may, though need not include grA and grB.

Secondly, when either grA or grB were deleted, then killer cells induced DNA fragmentation, though a double knockout abolished this response (Heusel et al., 1994; Simon et al., 1997). DNA fragmentation was also sensitive to pfn deletion (Simon et al., 1997). This indicated that DNA fragmentation during granule-mediated killing requires

grA or grB along with pfn. Notably, there has been a standing dogma that grB induces DNA fragmentation with faster kinetics than grA, thus providing a theoretical means of distinguishing these pathways. This was based on observations from the knockout killing assays that on a short-term scale (2-4 h), DNA fragmentation was grB dependent, while long-term (4-20 h) was mediated by both grA and grB (Heusel et al., 1994; Simon et al., 1997). Similarly, when purified granzymes and pfn were delivered to the T-lymphoma line Yac-1, grA was found to require 20 h in order to induce significant DNA fragmentation, while grB required only 2h (Shi et al., 1992a). However, more recent data has suggested that the relative importance of grA versus grB is dependent on the nature of the target. For instance, in the mouse lymphoma line L1210.3, DNA damage was exclusively grB-dependent; however in the T lymphoma line EL4.F15 and the fibrosarcoma line MC57G, there was partial dependence on both grA and grB (Pardo et al., 2002). Overall, this indicates that it is an oversimplification to assume that grB is the primary contributor to early onset DNA fragmentation.

In the end, the goal is a reliable method to distinguish grA- and grB-mediated killing events. Splenocytes derived from granzyme knockout mice are the most effective means of differentiating between these pathways, particularly when monitoring DNA fragmentation and chromium release. However, when this is not a practical option, tracking granzyme-specific pathway activation and/or cleavage products is a relatively reliable means of discernment.

1.2.4.2. Distinguishing granzyme- from Fas-mediated events

Since Fas, like the granzymes, can contribute to both DNA fragmentation and chromium release during killer cell attack, a means of discrimination is required. A

common approach has been based on the fact that Fas-mediated killing was distinguished from granule-mediated killing based on its calcium independence (Rouvier et al., 1993). Specifically, chelation of extracellular calcium with EGTA has been used in the assumption that this specifically blocked granzyme-mediated killing by preventing granule release. However, Fas-L is stored in granules, thus surface expression of Fas-L is dependent on degranulation (Bossi and Griffiths, 1999). As such, treatment with EGTA would not only prevent release of granzymes and pfn, but would also prevent Fas-mediated killing by sequestering the Fas-L within the granules. Clearly, EGTA is a poor means of distinguishing the Fas-mediated pathway from those of the granzymes.

As a side note, there is a further concern associated with calcium chelation. Calcium is a complex signaling molecule, serving as both a positive and pathological effector, affecting events ranging from protein expression through to cell death (reviewed in Brookes et al. (2004); Groenendyk et al. (2004)). While it is true that calcium signaling is affected by both intracellular and extracellular sources, chelating the extracellular calcium with EGTA could alter any number of metabolic events regulating both cell health and death responses. As such, chelation of calcium in killing assays might yield artifactual results.

Rather than using calcium chelation, a more reliable approach to measure granzyme-mediated killing is by pre-treating killer cells with concanamycin A (CMA). This agent neutralizes the pH in lysosomes and granules, which leads to loss of functional pfn within the granules (Kataoka et al., 1996). Thus, upon degranulation, active pfn is lacking and so cannot assist granzymes to kill the target cell. As such, observed

apoptotic activity in the presence of CMA may be considered granzyme- and pfn-independent, and likely Fas-mediated.

Alternatively, cells may be treated with a blocking antibody to the Fas receptor, which will prevent engagement of the Fas-L and downstream induction of apoptosis. All remaining cell death in the presence of the blocking antibody may be considered Fas-independent.

1.3. The Granule Proteoglycan, Serglycin

The proteoglycan SG is found in secretory granules, and is believed to assist the dense packing of granule components. SG is also secreted upon degranulation of CTL and NK cells, and remains complexed to some of the co-secreted cytotoxic effectors, suggesting a role outside the cell.

1.3.1. Serglycin

The proteoglycan SG is stored in the secretory granules of various hematopoeitic cell types (reviewed by Stevens et al. (1988); Kolset et al. (2004)). The peptide component has a distinct core domain that consists of repeating serine-glycine units (Bourdon et al., 1985; Bourdon et al., 1986). This composition of the core domain is the property to which SG owes its name, and is the attachment site for *O*-linked glycosaminoglycan (GAG) chains, all of which are sulfated, and therefore negatively charged. Glycosylation of SG is a cell type-specific process in that an array of GAG

types may be linked to the SG core. Chondroitin sulfate A (CS-A) is the basic GAG unit decorating SG synthesized by NK cells and activated CTL (Dvorak et al., 1983; MacDermott et al., 1985; Parmley et al., 1985). The molecular weight of SG proteoglycan secreted from the human NK cell line YT-Indy has been estimated to have a mean of 250 kDa (Raja et al., 2002). Notably, because regulation of GAG chain synthesis is imprecise, the molecular weight of proteoglycans isolated from any given cell population will be polydisperse.

1.3.2. Serglycin: a Role in Granule Packing

One of the roles proposed for SG is to aid in the packing and storage of cytotoxic granule proteins in CTL and NK cells. This hypothesis is first supported by the observation that in isolated granules, there is a stable interaction between SG and the cytotoxic granule factors, including grA (Stevens et al., 1988; Kamada et al., 1989), grB (Metkar et al., 2002), and pfn (Masson et al., 1990; Metkar et al., 2002). The nature of this interaction is predicted to be charge dependent, since high concentrations of salt disintegrate grA-SG complexes, while non-ionic detergents and urea have no effect (Stevens et al., 1988; Kamada et al., 1989). This prediction is consistent with the fact that SG is negatively charged due to the sulfated CS-A side chains, while the granzymes are positively charged (Masson and Tschopp, 1987).

A packaging function for SG has been tested further through the disruption of SG expression. Serglycin maturation has been disrupted by knockout of a critical sulfotransferase (Forsberg et al., 1999; Humphries et al., 1999), or in a more recent

publication, the SG gene itself has been deleted (Åbrink et al., 2004). In both cases, mature mast cells were impaired in their ability synthesize dense granules. The enzymatic activity of granule proteases was substantially suppressed in all instances, though the mRNA levels were unaffected (Forsberg et al., 1999; Humphries et al., 1999; Åbrink et al., 2004), suggesting SG regulates protease expression at a post-translational level. The exact causes of this defect remain unknown. One possible explanation implies that SG is required for trafficking to granules, so in the absence of SG, proteases are constitutively secreted. An alternate explanation is that SG may stabilize proteases within the confines of the granules, and that the absence of SG leads to proteolytic degradation of the granule cargo.

Notably, the hypothesized role of SG in granule formation has also been tested in CTL. In order to prevent synthesis of proteoglycan, CTL were incubated in the presence of PNP-xyloside, a molecule that competes with the proteoglycan peptide core for acceptance of the GAG side chains. Following 4 days co-culture with PNP-xyloside, CTL were used as killing effectors in a chromium release assay, but it was found that pfn lytic function was uncompromised (Masson et al., 1990). This suggested to the authors that intact SG proteoglycan did not play a critical role in maintaining pfn activity in the granules; however, several considerations must be made that might allow for such a result. Firstly, DNA fragmentation was never assessed; thus, the impact on the grA and grB has not been assessed. Secondly, data indicated that while intact proteoglycan was absent after PNP-xyloside treatment, 60-kDa GAG chains were detected in the granules (Masson et al., 1990). These GAG side chains might be able to functionally substitute for whole SG, since CS-A chains have been able to functionally substitute for intact SG and

form complexes with grB (Galvin et al., 1999). Thirdly, TCR stimulation in activated T cells has been found to induce granule protein synthesis *de novo*. Newly synthesized effector molecules are constitutively released and functionally act on target cells, rather than being directed to granules (Isaaz et al., 1995). If this pathway were activated in the PNP-xyloside-treated CTL, the newly synthesized granule proteins could effect a normal cytotoxic response. In conclusion, further work must be done to better assess the role of SG in cytotoxic granule formation.

1.3.3. Serglycin: a Role in Extracellular Activities

As well as its proposed role in granule packaging, SG might also play a role outside the cell following secretion. To begin, SG has been detected in the culture medium following degranulation of CTL and NK cells (Levitt and Ho, 1983; MacDermott et al., 1985). More interestingly, stable interactions have been detected between degranulated grA and SG (Masson et al., 1990), as well as grB and SG (Galvin et al., 1999). Two important observations have been made regarding these complexes. Firstly, grA-SG complexes were detected in the plasma of healthy patients (Spaeny-Dekking et al., 2000). Investigation of these complexes suggested that binding to SG protected grA against inactivation by protease inhibitors found in blood plasma (Spaeny-Dekking et al., 2000). Secondly, grB-SG complexes and pfn were functionally comparable to purified grB monomer and pfn in the induction of apoptosis (Metkar et al., 2002). Thus, binding to SG does not appear to have a deleterious effect on grB pro-apoptotic activity. Beyond these observations, the overall importance and impact of these complexes is still unclear.

1.3.3.1. Extracellular serglycin complexes: the impact of pH on stability

Given the differing pH between granules and the extracellular milieu, the proposal that SG remains bound to cargo following degranulation has important implications, Specifically, granules have an acidic pH, estimated at 5.4-5.5 (Burkhardt et al., 1990; Masson et al., 1990), while the extracellular environment is in the neutral range. This differential pH is important because the SG–cargo interaction is charge-based, and charge may, though need not be altered by a change in pH. For example, the mouse mast cell proteases (mMCP) 6 and 7, both bind tightly to heparin-modified SG at the acidic pH of mast cell granules. Upon degranulation, where the molecules encounter a neutral environment, key histidine residues on the surface of mMCP-7 lose their positive charge, altering the nature of its SG-binding domain, and causing its dissociation (Ghildyal et al., 1996). In contrast, mMCP6 binds SG in a manner dependent primarily on lysine and arginine residues, which do not alter charge in the acid to neutral switch. The result is that this enzyme remains tightly bound to SG at neutral pH after secretion (Ghildyal et al., 1996).

There is supporting evidence to suggest these sorts of binding and dissociating events also occur with proteins secreted from the granules of CTL and NK cells. Granules were isolated from a mouse CTL cell line, these were treated with buffers of pH 5.5 and 7.4, and then grA and pfn proteins were tracked in the supernatants and granule pellets. Both proteins associated with SG in the granule pellet at pH 5.5. However, at pH 7.4, the majority of pfn was found in the supernatants, while most of grA remained associated with the pellet (Masson et al., 1990). As a further exploration, granzymes A, D and E were fractionated over a CS-A affinity column at pH 5.5 and 7.4, and eluted with

step gradients of 150, 300, and 600 mM sodium chloride. Overall, the elution patterns revealed that all granzymes bound with decreased stability at pH 7.4 compared to pH 5.5 (Masson et al., 1990). However, grA was effectively retained on the column under extracellular conditions, that is pH 7.4 and 150 mM salt (Masson et al., 1990). In contrast, only half of each of the granzyme D and E pools was retained at pH 7.4 and 150 mM salt (Masson et al., 1990). These data illustrate that upon degranulation, the granule molecules equilibrate with varying degrees between free and SG-bound forms.

Some work has also been done to characterize the stability of grB-SG complexes following degranulation. Strikingly, claims have been made that grB is exclusively SG-bound in degranulate material, since no free grB was detected (Galvin et al., 1999). This claim has received some support from kinetic studies by surface plasmon resonance, since the data did not reveal a distinct difference in the stability of grB-SG complexes between pH 5.5 and 7.4 (Raja et al., 2002). A further point of interest from these data was that after a drop in grB concentration, very little grB dissociated from SG and some even re-bound (Raja et al., 2002). This suggests that the dilution that is the result of degranulation will have a minimal effect on grB-SG complex stability.

There remain a few points to suggest the pH change upon degranulation may induce some grB-SG dissociation. Analysis of grB binding to CS-A at pH 5.5 and 7.4 showed a greater complex formation at acidic pH (Galvin et al., 1999). Likewise, when the binding capacity of SG for grB was estimated, it was found that 51 molecules of grB could bind a single SG at pH 5.5, while only 32 could bind at pH 7.4 (Raja et al., 2002). Despite these inferences, the degree to which grB and SG will dissociate upon degranulation is unknown.

1.3.3.2. Extracellular serglycin complexes: binding mixed cargo

An interesting observation from isolated granules has been that grB and pfn are physically linked through SG association. This conclusion is based on two separate methods: co-immunoprecipitation (Metkar et al., 2002), and a gel shift assay subsequently analyzed by immunoblotting (Raja et al., 2002). From these observations, it has been inferred that SG may bind a mixture of cargo during packaging in granules. It then follows that in degranulate material, where cargo remain bound to SG, there will be mixed complexes. If one molecule is recognized by a specific receptor on the surface of a target cell, and if the mixed complex remains intact during uptake, then a whole array of cytotoxic effector molecules might be internalized. Though this hypothesis is compelling, there is no evidence to further support or argue it.

1.4. The Entry Mechanism of Granzymes into Target Cells

Both granzyme A and B cleave specific substrates in the cytoplasm of target cells, directly implying that these cytotoxic molecules must gain access to the cytoplasm. The pfn pore translocation model (described in **section 1.2.2.3.**) has been proposed to account for granzyme internalization; however, data to support this model have been lacking. Rather, accumulating data from grB studies suggest an endocytic mechanism of granzyme uptake, followed by pfn-dependent release of grB from the endosome. Some data have suggested that the uptake event involves a specific receptor, thus some have aimed to identify the receptor. Other studies have argued in favour of an endocytic event

independent of a specific receptor. Based on the current controversy in the literature, no clear model has emerged to describe grB endocytosis.

1.4.1. Granzyme Uptake Occurs by Endocytosis

The model proposing granzyme endocytosis has been supported from two separate angles. Firstly, it has been found that grB, which has served as a prototype for granzymes in general, can enter cells independently of pfn via an endocytic route. The second form of support for this model has been the finding that pfn activity may not directly mediate grB translocation through the plasma membrane, but rather is important for its release from an endosome into the cytoplasm.

1.4.1.1. Evidence for granzyme uptake by endocytosis: granzyme B studies

It was first hinted that granzyme B uptake might occur via an endocytic mechanism when grB and pfn-mediated apoptosis was inhibited by cytochalasin B, an inhibitor of actin polymerization and thus of early endocytic events (Shi et al., 1992b). However endocytic uptake of grB only received serious attention when it was observed that grB could be internalized by cells in the absence of pfn, though induction of apoptosis was wholly reliant on pfn (Froelich et al., 1996). If target cells were first pulsed with grB, then washed to remove the excess, subsequent addition of pfn induced apoptosis, whether the addition was immediate or after a recovery period (Froelich et al., 1996). As well, internalization of radiolabeled grB was detected when cells were incubated with grB in the absence of pfn, at room temperature or 37°C (Froelich et al., 1996). Together, these data implied that, independently of pfn, grB can interact

specifically with a cell, be endocytosed, and still retain its apoptotic activity. Another important implication was that if pfn is also internalized, it need not enter the cell in the same vesicle as grB in order to cause apoptosis. If the two must finally localize to a common vesicle to cause grB release, this situation can occur via vesicular fusion.

Notably, grB-SG uptake has been tested in a similar manner. Cells were pulsed with grB-SG in degranulate materials, washed, and then treated with adenovirus (AdV), which can functionally substitute for pfn. Targets treated in this way still underwent apoptosis (Galvin et al., 1999). This suggested that SG-bound grB is still able to interact specifically with the surface of target cells, though these data did not address whether SG dissociates from grB upon receptor binding, or whether the whole complex binds to the cell surface.

The grB endocytosis hypothesis has been supported through uptake studies using fluorescently labeled grB in the absence of pfn (Shi et al., 1997; Pinkoski et al., 1998). Under these conditions, punctate fluorescent staining was visible in treated cells (Pinkoski et al., 1998), suggesting grB is enclosed within endosomes. Upon addition of pfn, intense punctate staining was lost, but the fluorescence distributed diffusely throughout the cytoplasm, and within 20 min, began to accumulate in the nucleus (Pinkoski et al., 1998). These observations were consistent with pfn-assisted release of grB from the endosome into the cytoplasm. Also, the grB uptake was found to be energy-dependent (Shi et al., 1997), indicating an active rather than a passive internalization event.

Additional uptake studies by confocal and electron microscopy monitored the endocytic route of grB. Early endocytic events have been tracked through early

endosomes labeled with rab4 and rab5 (Pinkoski et al., 1998). In more recent studies, after delivery of grB-SG complexes, during late stage trafficking, grB was found to accumulate in vesicles labeled with lamp-1, a marker of late endosomes and lysosomes (Dressel et al., 2004a).

1.4.1.2. Evidence for granzyme uptake by endocytosis: perforin studies

The first suggestion that pfn does not function as a granzyme translocating pore was when the detergents Triton X-100 or digitonin could not functionally replace pfn in grB-mediated apoptotic assays (Shi et al., 1997). Likewise, ionomycin, the calcium ionophore could not replace pfn (Shi et al., 1997). Thus neither could pore-forming molecules mediate grB translocation, nor was calcium influx sufficient, indicating this is not a likely means by which pfn is acting. Along similar lines, the pore-forming complement complex could not functionally replace pfn in delivery of grB (Browne et al., 1999), despite its strong structural homology to pfn (Liu et al., 1986; Shinkai et al., 1988). In contrast, pfn activity could be substituted by AdV (Froelich et al., 1998) and listeriolysin O (Browne et al., 1999), which was particularly interesting since both are known to disrupt endosomes. Also, the membrane disrupting agents streptolysin O (SLO) and pneumolysin O (PLO), which are structurally related to listeriolysin O, were functional replacements for pfn (Browne et al., 1999).

An important hypothesis derives from the fact that pfn may be functionally replaced, and that delivery of grB is sufficient to induce apoptosis. This indicates that the primary role of pfn is indeed to deliver pro-apoptotic molecules into the cytoplasm of target cells, rather than having its own intrinsic apoptotic function. The hypothesis is

further supported by observations that microinjection of grB into the cell cytoplasm is sufficient to induce apoptotic traits in injected cells (Pinkoski et al., 1998).

The pfn-pore translocation model has been further supported by the finding that exceedingly high concentrations of pfn would be required in order to form a pore of sufficient size to allow passage of grB. Though 1000-1500 U/mL pfn lysed at least 80% of cells, 5000 U/mL were required to form pores of sufficient size to deliver grB or grA (Browne et al., 1999). As a stark contrast, a pfn concentration of 300-500 U/mL, which causes less than 10% lytic damage, was more than sufficient to assist grB in inducing apoptosis (Browne et al., 1999). While an important unknown quantity is the local concentration of pfn and granzymes within the immunological synapse, even within this condensed volume it seems unlikely that sufficient pfn would be released to cause the extreme membrane damage required to allow direct granzyme translocation.

After the initial proposal that grB entered cells by an endocytic mechanism, a new model for pfn action was required. One such hypothesis proposed that pores formed at the cell surface, and then were internalized as a damage repair response (Morgan et al., 1987). Internalization of pfn pores would either allow co-internalization of grB and pfn in one vesicle, or allow fusion of two vesicles to bring grB and pfn together. More recent data has emerged, however, to suggest that lytic pfn pores may never form at the cell surface when assisting grB-mediated apoptosis. For instance, when cells were treated with pfn, the population of permeabilized cells did not decrease over time (Metkar et al., 2002), suggesting that these cells did not, in fact, invoke a membrane repair mechanism. A more important finding, however, was obtained when grB and pfn-mediated killing was monitored in the presence of the DNA-chelating fluorescent dyes Yopro-1 and

propidium iodide (PI). The principle of the assay was based on the fact that Yopro-1 is able to enter semi-permeabilized apoptotic cells, but PI is excluded until the membrane is fully permeabilized, either by pfn or by late stage apoptosis. In this way, one would predict that if grB uptake depended on transient pfn pores on the plasma membrane, then cells would be stained with PI. However, a significant population of cells were PI-negative and Yopro-1 positive (Metkar et al., 2002). This suggested that pfn had not formed pores on the surface of these cells, yet pfn had effectively assisted with the entry of grB into the cells to induce apoptosis. This then suggested that pfn possesses a distinct primary mechanism involved in granzyme delivery, and that its well-known lytic activity may in fact be secondary.

In support of a distinct, non-lytic pfn activity, it was found that pfn-assisted release of grB from endosomes was delayed by bafilomycin or ammonium chloride, treatments that prevent endosomal acidification (Browne et al., 1999). Notably, pfn lytic activity is optimal around neutral pH (Young et al., 1986a), but since acidification seemed to speed release of grB from the endosome, this further suggests that the lytic properties of pfn are secondary to those involved in grB release from endosomes.

To add a final piece of evidence supporting a non-lytic pfn mechanism, pfn activities have been compared while pfn was in its free or SG-bound state. Indeed, it has been found that pfn preferentially dissociates from SG in extracellular conditions (Masson et al., 1990), however a small but significant pool of pfn-SG complexes can be formed under these conditions (Metkar et al., 2002). When these complexes were generated, it was found that they had reduced membranolytic activity, compared to free pfn (Metkar et al., 2002). This was consistent with previous findings that showed GAGs,

including CS-A, inhibited pfn lytic activity (Tschopp and Masson, 1987). Strikingly, when pfn-SG was tested for its ability to deliver grB to induce apoptosis, activity was comparable to free pfn (Metkar et al., 2002). Bear in mind that pfn lytic activity was impaired when complexed to SG, thus that activity was not critical when pfn assisted grB delivery. As such, pfn membranolytic activity must not be critical in mediating granzyme delivery.

Together these data suggest a new hypothesis, that pfn is acting internally, to release granzymes from an endosome, and that this activity does not involve pre-formation of pfn pores on the plasma membrane. Given that a substantial amount of pfn is required to form pores of sufficient size to allow direct grB translocation (Browne et al., 1999), it seems unlikely pfn disrupts endosomes through pore formation. Rather, a more plausible model that has been proposed is that the presence of pfn interferes with normal fusion events of endosomes, destabilizing the newly-formed endosome overall, and thereby allowing release of the contents (Metkar et al., 2002).

1.4.2. Granzyme Uptake Model: Receptor-Dependent Endocytosis

The grB endocytosis hypothesis naturally implies that endocytosis might be receptor-mediated. When grB binding and uptake kinetics were studied, it was found that radiolabeled grB could bind to the cell with high specificity, in a concentration-dependent and saturable manner, all of which are critical factors suggesting the existence of a specific grB receptor (Froelich et al., 1996). Titrations revealed that cell-associated grB increased proportionally to grB concentration, and these associations were mediated by

both specific and non-specific interactions. The high-affinity, specific interactions were identified through competition with unlabeled grB, and were saturated at 1.5 μg/mL grB (Froelich et al., 1996). The affinity of specific binding sites was estimated at a K_d of approximately 10 ng/mL, and numbered 3×10^4 on the surface of the Jurkat T-cell line (Froelich et al., 1996). The non-specific grB-cell interactions increased linearly even in the presence of competing unlabeled grB, were detectable after 700 ng/mL grB, and by 1.5 μg/mL grB, approximately half of the binding events could be attributed to non-specific interactions (Froelich et al., 1996).

Further support for a specific grB receptor came from experiments in which cells were pulsed with grB at 4°C to allow specific binding, but prevent uptake of grB. Cells were then washed with neutral media or with acidic citrate buffer, and then pfn was added (Pinkoski et al., 1998). Those cells washed with neutral media underwent apoptosis, suggesting a high affinity interaction between grB and the target cells. In contrast, the cells subjected to acid wash displayed a significantly reduced level of apoptosis, suggesting that the specific grB binding was disrupted by acidic pH, presumably by disrupting a charge-dependent interaction.

This hypothesis that grB uptake is receptor-dependent has yielded numerous efforts to characterize both the pathway and the receptor for endocytosis. Models tested so far include two putative grB receptors, namely the cation-independent mannose 6-phosphate receptor (CI-MPR) and heat shock protein 70 (Hsp70). In contrast, some data have implied that uptake may actually be receptor-independent.

1.4.2.1. A putative granzyme B receptor: the cation-independent mannose 6-phosphate receptor

One model to describe the mechanism of grB endocytosis has surrounded the CI-MPR as a putative receptor. This is an enticing receptor to consider for this pathway because of the M6P modification found on grB (Griffiths and Isaaz, 1993), as well as grA (Burkhardt et al., 1989). The CI-MPR has been well characterized in its role to traffic M6P labeled lysosomal enzymes, from the *trans*-golgi network or the cell surface, into the late endosome (reviewed by Ghosh et al. (2003)). As such, it might operate analogously in grB uptake from the surface of target cells. Notably, there is a related receptor, the cation-dependent mannose 6-phosphate receptor (CD-MPR) that is involved in similar trafficking activity, except that the slightly alkaline pH of the cell surface prevents effective ligand binding by this receptor. As such, the CD-MPR is a less likely candidate for grB-mediated endocytosis. The CI-MPR is even more interesting because it may be a tumour suppressor (Ghosh et al., 2003). If the receptor were down-regulated at the cell surface, this could interfere with granzyme uptake and therefore decrease target cell susceptibility to cytotoxic attack. Notably, the proposed tumour suppressor role might also be due to non-trafficking functions of the CI-MPR. Discussion of these functions is beyond the purpose of this thesis and may be reviewed elsewhere (Ghosh et al., 2003).

The involvement of mannose 6-phosphate receptors (MPRs) in killer cell-mediated cytotoxicity was first entertained even before it was known that grA (Burkhardt et al., 1989) and grB (Griffiths and Isaaz, 1993) possess the M6P modification. NK-mediated cytotoxicity was tested in the presence of MPR blockers, namely MPR-specific

antibodies and soluble M6P (Haubeck et al., 1985). Likewise, for use as effectors in cytotoxic assays, NK cells (Haubeck et al., 1985) or CTL (Griffiths and Isaaz, 1993) were derived from I-cell patients because this condition prevents correct synthesis of the M6P tag, including on grB and grA (Griffiths and Isaaz, 1993). None of these blockers of MPR-binding were able to inhibit a chromium release response. Unfortunately, DNA fragmentation was not assessed. Based on this, it was initially concluded that MPRs had little to do with cytotoxic mechanisms of killer cells. Consider, however, that the chromium release assay would not specifically monitor the activities of the M6P-labeled molecules, granzymes A and B. Rather, the assay would indicate activity of pfn, the molecule that lacks a M6P tag. Thus these early studies may be considered inconclusive with respect to MPR-mediated uptake models for grA and grB.

More recently in has been suggested that the M6P tag on grB is a functional and critical motif, both for binding to cells and to mediate apoptosis (Motyka et al., 2000). This was because binding of grB to target cells, as well as grB and AdV-mediated apoptosis were both inhibited by removal of the phosphate group from the M6P tag (Motyka et al., 2000). Soluble M6P was also an effective competitive inhibitor of these events (Motyka et al., 2000). The importance of these first observations was questioned by subsequent findings that M6P only partially blocked uptake of grB in a HeLa-derived cell line (Trapani et al., 2003). Thus, these later data suggested that while an MPR-dependent grB uptake pathway existed, there was also an MPR-independent pathway.

Next, the relative importance of each MPR was assessed in binding of, as well as killing by grB. The experimental system for these studies involved cell lines derived from a mouse L-cell fibroblast sub-line that lacked surface expression of the CI-MPR

(CI-MPR$^-$; Gabel et al., 1983). Transfection of the parental cell line yielded CI-MPR- or CD-MPR-overexpressors (CI-MPR$^+$ and CD-MPR$^+$, respectively; Watanabe et al., 1990). While strong grB binding was detected on either CI-MPR$^+$ or CD-MPR$^+$ cell lines, induction of DNA degradation by grB and AdV occurred only in the CI-MPR$^+$ cell line (Motyka et al., 2000). In contrast, both the CI-MPR$^-$ and the CD-MPR$^+$ lines were insensitive to attack by grB and AdV (Motyka et al., 2000). This suggested that the CD-MPR was not involved in grB uptake and killing, but more importantly, that the CI-MPR was. Contrasting evidence was presented when the CI-MPR$^+$ or CI-MPR$^-$ lines were challenged with grB and pneumolysin O. Now, cell viability was independent of CI-MPR expression at high levels of grB (1.6 μg/mL), but was dependent at a low level of grB (400 or 100 ng/mL; Trapani et al., 2003). Also, induction of chromium release, at 1.6 μg/mL grB required the CI-MPR at early time-points, but the receptor was less critically involved as time progressed (Trapani et al., 2003). These findings did not deny the presence of a CI-MPR-dependent grB uptake pathway. Rather, they implied that an alternate, albeit kinetically slower, CI-MPR-independent route is also present, and may be sufficient in grB-mediated killing.

Eventually, the CI-MPR$^+$ and CI-MPR$^-$ cell lines were used as targets for alloreactive CTL effectors. Chromium release response was indistinguishable in the two lines, though DNA fragmentation was dependent on CI-MPR expression (Motyka et al., 2000; Trapani et al., 2003). Further, when these cells were treated with splenocytes from grB knock-out mice, DNA-fragmentation was abolished (Trapani et al., 2003), suggesting that in these cell lines, grB is the predominant factor inducing DNA fragmentation. This set of data, therefore implies that the CI-MPR may have a critical impact on grB-induced

apoptotic responses. However, since chromium release was CI-MPR-independent, pfn and other granule factors may still be able to act independently of the CI-MPR to eliminate the target cell (Simon et al., 1997).

Once again, a concern was raised against these data, now due to the use of the cell lines derived from CI-MPR$^-$ L cell fibroblasts (Dressel et al., 2004a). The criticism was that the death responses in these cell lines are poorly characterized overall, and may not be normal. For instance, in the parental cell line, along with the CI-MPR, expression and/or function of other unknown factors could be altered. Also, in the transfected lines, expression of the CI-MPR above normal levels might lead to artifactual responses.

In order to address this issue, target cells were derived from CI-MPR and/or CD-MPR knockout mouse lines of defined genetic backgrounds (Dressel et al., 2004a; Dressel et al., 2004b). Now, in response to alloreactive CTL, neither chromium release nor DNA fragmentation were affected by MPR expression. Since DNA fragmentation was both EGTA-sensitive (Dressel et al., 2004a; Dressel et al., 2004b) and caspase-dependent (Dressel et al., 2004a), it was assumed that this death response was grB-dependent. Thus, it was concluded that CI-MPR-mediated uptake was not a critical grB pathway in granule-mediated killing. The reasons for the different outcome have not yet been explored in an experimental system. However, the data were supported by the observation that purified grB or grB-SG complexes could be internalized to comparable degrees in both wild type and CI-MPR-deficient cells (Dressel et al., 2004a).

Finally, the issue of CI-MPR-dependent uptake has also been debated through allogeneic transplant rejection models. In the first example of this experimental system, graft rejection was CI-MPR-dependent (Motyka et al., 2000), which lent further support

to the CI-MPR-mediated granzyme uptake model. The subsequent inability to reproduce these results has been invoked in arguments against the CI-MPR-mediated granzyme uptake model (Trapani et al., 2003; Dressel et al., 2004b). However, a recent publication has shown that T-cell-mediated lesions of kidney allograft rejection were not dependent on pfn, grA or grB (Halloran et al., 2004). Overall, this finding suggests that use of allotransplant to study mechanisms of grA, grB and pfn-mediated killing is problematic. Thus the allotransplant experiments to test CI-MPR-dependent uptake of grB reveal nothing either way about the mechanism of interest.

Ultimately, in the debate surrounding CI-MPR-mediated uptake of grB, the data stand as controversial and inconclusive. Potential causes for the observed differences must first be explored in order to clarify their meanings.

1.4.2.2. A putative granzyme B receptor: heat shock protein 70

Another receptor model emerged when grB was identified as an Hsp70-binding protein (Gross et al., 2003). This model is intriguing because Hsp70 is up-regulated and expressed on the cell surface in some forms of cancer, so this could serve as a useful means of grB uptake in cells displaying this stress marker (Multhoff et al., 1997). The Hsp70 grB-receptor model was supported by several observations. First grB interacted with the Hsp70 domain that is expressed on the cell surface, rather than binding via the chaperone domain (Gross et al., 2003). Secondly, Hsp70-dependent uptake of grB was detected (Gross et al., 2003). Thirdly, in the absence of pfn, grB was able to induce low, though significant levels of apoptosis in an Hsp70-dependent manner, after 24 h incubation (Gross et al., 2003). And finally, the presence of Hsp70 on the surface of target cells enhanced chromium release in response to NK cells that had been stimulated

with an Hsp70 peptide (Gross et al., 2003). And since ionic channels formed when Hsp70 was integrated in vesicle membranes, it was proposed that grB translocates through an Hsp70 channel (Gross et al., 2003). Beyond these findings, there has been no further investigation of either the properties or the relevance of Hsp70-grB interactions. It should be noted, however, that grA also has been found to interact with Hsp70 (Beresford et al., 1998), though neither the nature nor the function of this interaction have been characterized.

1.4.2.3. A putative granzyme B uptake pathway: receptor-independent

Amid the controversy over grB receptors, a third model has proposed that endocytosis actually occurs independently of a receptor. In support of this model, not only a CI-MPR-independent mechanism has been suggested (as discussed in **section 1.4.2.1.**), but also a dynamin-independent mechanism (Trapani et al., 2003). Though dynamin-dependent and, therefore, clathrin-mediated endocytosis were blocked, grB could still be internalized, and could effectively induce chromium release in the presence of pfn (Trapani et al., 2003).

The receptor-independent model has found even more striking support through the study of grB binding to cell-surface heparan sulfate. Strikingly, grB could bind to cells via the heparan sulfate displayed on cell surfaces, but blocking this interaction was an ineffective means of preventing apoptosis (Kurschus et al., 2005). Specifically, grB binding sites could be competed by several negatively charged proteases, such as granzyme K or non-labeled inactive grB (Kurschus et al., 2005). Alternatively, grB binding could be prevented by removing heparan sulfate from the cell surface, either by treatment with heparinase III, or by treating cells for 36 h with 50 mM sodium chlorate

(Kurschus et al., 2005). Unexpectedly, however, these treatments had no impact on apoptosis induced by grB and SLO, or by NK cells (Kurschus et al., 2005). An important note was that in order to simplify the study, the chosen grB source was recombinant, since grB would then lack the M6P and not be able to bind to MPRs. Therefore, since killing was achieved in the absence of detectable grB binding, it has been proposed that uptake of grB may occur by a receptor-independent mechanism.

1.4.3. Uptake of Granzyme A

No studies have been directed at the uptake mechanism of grA, therefore models are based on what is known for grB. Regardless of the fact that such assumptions are unreliable, it is fair to say that little is truly known about potential grA uptake events, given the controversy surrounding grB. All that can be said is that grA possesses a M6P moiety (Burkhardt et al., 1989), so that it is a legitimate candidate for CI-MPR-mediated uptake.

1.5. Aims of the thesis

The aim of this thesis is to address some of the controversy surrounding grB uptake. Further, the impact of SG during uptake is newly explored, since SG forms a physiological complex with grB and grA.

The specific objectives addressed are as follows:

1) Assess whether dynamin is critical in grB uptake and subsequent death events, and whether these events are impacted by the presence or absence of SG.
2) Further test the hypothesis that grB uptake occurs via the CI-MPR, with a particular focus on the impact of SG and other GAGs.
3) Characterize the grA- and grB-SG complexes with respect to stability, ability to bind to the CI-MPR, and mixed binding of grA and grB proteases on SG.

Chapter 2

Materials and Methods[1]

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[1]Portions of this chapter have been published. Kirstin Veugelers, Bruce Motyka, Christine Frantz, Irene Shostak, Tracy Sawchuk, and R. Chris Bleackley, 2004. The granzyme B-serglycin complex from cytotoxic granules requires dynamin for endocytosis. Blood. 103:3845-3853. © The American Society of Hematology.

2.1. Chemical Reagents

Unless otherwise specified, chemical reagents were available from Sigma-Aldrich Canada Ltd. (Oakville, ON).

2.2. Cells

2.2.1. Targets and control cell lines

HeLa-DynWT (HtTa- wild-type dynamin) and HeLa-DynDN (HtTa- K44A dynamin) cells (Damke et al., 1994; Damke et al., 1995) were generated in the laboratory of Dr S.L. Schmid (Scripps Research Institute, La Jolla, CA), and were kindly provided by Dr B. Finlay (University of British Columbia, Vancouver, BC), with permission. In brief, these cells were derived by transfection of the dynamin 1 wild type or K44A mutant gene, respectively, into a stable HeLa cell line expressing the dimeric tetracycline regulatable transcription activator. These cells were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen Canada Inc., Burlington, ON) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT), 100 U/mL each of penicillin and streptomycin (Invitrogen), 400 μg/mL active G418 (geneticin; Invitrogen), 200 ng/mL puromycin, and 1 μg/mL tetracycline-hydrochloride (tet). To induce expression from the transfected dynamin constructs, tet was omitted from the medium, and cells were allowed to culture for 48 h in its absence, with a change of media after 24 h. Also, both induced and parallel control cultures were incubated in their normal growth media, except for a substitution of 10% Tet system approved FBS (BD Biosciences, Mississauga, ON).

Cation-independent mannose 6-phosphate receptor (CI-MPR)-overexpressing (CI-MPR$^+$) and –deficient (CI-MPR$^-$) cells were obtained from Dr W. Sly (Saint Louis University School of Medicine, St Louis, MO). These cell lines were derived from an L cell line deficient in CI-MPR, by transfecting with either a human CI-MPR expression construct or empty vector, respectively (Gabel et al., 1983; Watanabe et al., 1990). Cultures were maintained in DMEM supplemented with 10% dialyzed FBS (Hyclone), 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine (Invitrogen) and 3.2 μM methotrexate, and 100 U/mL each penicillin and streptomycin.

The Jurkat T cell human lymphoma line (available from American Type Culture Collection (ATCC), Manassas, VA) was maintained in RHFM (RPMI 1640 medium, supplemented with 10% FBS, 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 1 mM sodium pyruvate, 100 μM 2-mercaptoethanol, and 100 U/mL each of penicillin and streptomycin).

L1210 (H-2^d) were a subclone of the original mouse lymphocyte leukemia line (available from ATCC), selected for resistance to Fas stimulation (Atkinson et al., 1998). Cells were maintained in RHFM.

Concanavalin A (ConA) stimulated lymphoblasts were splenocytes derived from Balb/c mice (H-2^d), and were stimulated 48 h with 5 μg/mL ConA in CTL media (RMPI 1640 media supplemented with 10% FBS, 90 U/mL interleukin-2 and 100 U/mL each of penicillin and streptomycin).

2.2.2. Effector cells

Human lymphocytes were isolated from peripheral blood, and cytotoxic T lymphocytes (CTL) were obtained as described previously (Atkinson et al., 1998). Briefly, lymphocytes were stimulated with irradiated Epstein-Barr virus-transformed RPMI-8666 cells. The expanded CTL population was maintained in CTL media, and was re-stimulated on a weekly basis.

Alloreactive CTL from wild type, $grB^{-/-}$, $grA^{-/-}/grB^{-/-}$, or $pfn^{-/-}$ mice, all with a mixed genetic background of C57BL/6 and 129 ($H\text{-}2^b$) were generated as previously described (Atkinson et al., 1998). Briefly, mouse splenocytes were stimulated with irradiated Balb/c ($H\text{-}2^d$) splenocytes. The expanded CTL population was maintained in CTL media, and was re-stimulated on a weekly basis. Note that prior to killing assays, there was no re-stimulation of effectors with anti-CD3 antibody.

2.3. Pro-Apoptotic Reagents

2.3.1. Granzymes

2.3.1.1. Enzymatic detection of granzymes A and B

Enzymatic activity of grA and grB were detected by the cleavage of BLT (N^{α}-benzyloxycarbonyl-L-Lys-thiobenzyl) ester and BAADT (butoxylcarbonyl-Ala-Ala-Asp-thiobenzyl) ester (Enzyme Systems, Livermore, CA) substrates, respectively (Pasternack and Eisen, 1985; Odake et al., 1991). Reactions were performed in 100 mM HEPES, pH 7.5, 10 mM calcium chloride, and included 200 µM BLT ester, 200 µM DTNB (5,5'-

dithiobis (2-nitrobenzoic acid)) and 4% dimethyl sulfoxide (DMSO), or 100 μM BAADT, 100 μM DTNB and 2% DMSO. Reactions were incubated at room temperature for 10 to 30 min.

Alternatively, grB activity was detected by the cleavage of IEPD-pNA (acetyl-Ile-Glu-Pro-Asp-paranitroanilide; Kamiya Biomedical Company, Seattle, WA; Ewen et al., 2003). Sample reactions were prepared in 50 mM HEPES, pH 7.5, 10% (w/v) sucrose, 0.1% (w/v) CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), and 5 mM dithiothreitol containing 200 μM IEPD-pNA and allowed to incubate at 37°C for 1 to 20 h.

The enzymatically cleaved products were quantified at A_{405} using a Multiskan Ascent spectrophotometer (Thermo Lab-systems, Helsinki, Finland). To estimate granzyme concentrations, samples were compared to a 2-fold dilution standard curve of granzyme, ranging from 2000 to 31 ng/mL, and interpolated from a non-linear regression curve (x=log[granzyme], y=A_{405}) using Graphpad Prism software, version 3.02 (Graphpad Software, Inc., San Diego, CA).

2.3.1.2. Purification of granzyme A

GrA was purified essentially as previously detailed (Hanna et al., 1993) from the NK-like cell line NK-92-MI (ATCC). In brief, 1-3 × 10^9 cells were disrupted by nitrogen cavitation, and a crude granule pellet was obtained by differential centrifugation. Membrane proteins were removed by serial extraction steps in 0.5% (v/v) Triton X-100 and low salt (25 mM sodium chloride). SG-bound proteins in the pellet were solubilized with an acidic hypertonic saline solution (390 mM sodium chloride, 20 mM sodium

acetate, pH 4.5, 2 mM calcium chloride), then loaded on a cation exchange column (5 mL HiTrap SP; Amersham Biosciences Corp., Piscataway, NJ) and eluted with a linear gradient from 25 mM to 1 M sodium chloride over 15 column volumes (1 mL/min elution rate). Fractions were tested for grA and grB activity by cleavage of BLT and BAADT ester substrates, respectively. Peak samples containing grA activity and lacking grB activity were pooled, and, when necessary, were concentrated on a 30-kDa cut-off Amicon Ultra-4 centrifugal filter (Fisher Scientific International, Nepean, ON). Aliquots were stored at –80°C.

2.3.1.3. Purification of granzyme B

Human grB was purified from the cytotoxic granules of YT-Indy cells as described (Caputo et al., 1999). Briefly, 2×10^9 cells were disrupted by three freeze-thaw cycles (-70 to 37°C), cellular debris was removed by centrifugation, and the resultant granule suspension was disrupted with 1% Triton X-100. Materials were sequentially loaded onto a heparin affinity column (5 mL HiTrap Heparin; Amersham), then a cation exchange column (5 mL HiTrap SP; Amersham), each eluted with a linear gradient of 0 to 1 M sodium chloride. GrB activity in eluted fractions was monitored by cleavage of BAADT ester, and peak fractions were pooled, aliquoted, and stored at –80°C.

2.3.1.3.1. Direct conjugation of Alexa 488 to grB

GrB-containing HiTrap SP fractions were pooled, and concentrated over a Centricon YM-10 centrifugal filter (Fisher), then grB was derivatized using an Alexa Fluor 488 (A488) protein labeling kit (Invitrogen). GrB-A488 was resolved from unbound dye by passing the material through a Sephadex G-25 HiTrap Desalting Column

(Amersham) with phosphate-buffered saline (PBS). GrB enzymatic activity was monitored by cleavage of IEPD-pNA.

2.3.1.3.2. Forming glycosaminoglycan-grB complexes

Heparan sulfate (250 µg/mL), heparin (100 µg/mL; Hepalean, Organon Teknika, St. Laurent, Quebec), or chondroitin sulfate A (50 µg/mL) were incubated with grB in DMEM and 0.1% (w/v) bovine serum albumin (BSA) for 60-90 min at 37°C.

For filter fractionation of complexes, materials were loaded on a YM-100 Microcon centrifugal filter (Fisher), and centrifuged at 12 000 × *g* until the filter was dry. Retained materials were then recovered in fresh media, in a volume equivalent to the starting material.

2.3.1.4. CTL degranulation and filter fractionation of degranulate

To obtain the degranulate material (chapter 3 and 5), anti-human CD3, clone HIT3a (BD Biosciences) was diluted in carbonate coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) and allowed to coat 6-well plates at 5 µg/mL antibody for 1 h at 37°C (chapter 3), or 12-well plates at 2 µg/mL antibody overnight at 4°C (chapter 5). Wells were washed extensively with PBS, then received 0.5 mL of 10^7 c/mL human CTL (hCTL) in degranulation media (Phenol Red-free RPMI (Invitrogen), 20% (v/v) PBS), to incubate 4 h at 37°C. Alternatively (chapter 4), anti-human CD3, clone OKT3 (received from Dr K. Kane, University of Alberta, Edmonton, AB), was diluted in PBS to 1 µg/mL, and allowed to coat 96-well plates for 1 h at 37°C. Wells were washed with PBS, then received 10^7 cells/mL hCTL in DMEM with 0.1%

BSA, to incubate 4 h at 37°C. In either case, the culture medium was recovered to collect the degranulate material.

In a similar manner, CTL degranulate was obtained by stimulation with target cells. CTL and Jurkats were co-suspended in degranulation media (5×10^6 cells/mL, each) to induce degranulation. Cell mixtures were incubated for 4 h at 37°C, then the degranulate material in the culture medium was collected for analysis.

To fractionate CTL degranulate, materials were concentrated over a Microcon YM-100 centrifugal filter at $1000 \times g$ until a control filter with degranulation media ran dry. When the high molecular weight granzyme-SG complexes were the desired material, the high molecular weight degranulate material (HighDegran; > 100 kDa) retained on the filter was recovered for further use.

2.3.2. Perforin and other lytic agents

Pfn and isolated granules, prepared from the rat NK-like line RNK-16 (Winkler et al., 1996), were kindly given by Dr D. Hudig (University of Nevada, Reno, NV). Notably, both pfn and isolated granule stocks contain 1 M sodium chloride. Human replication deficient AD type 5 d170-3 (AdV) was purified in our lab as described (Bett et al., 1994). Streptolysin O (SLO) was kindly obtained from Dr Sucharit Bhakdi (Institute of Medical Microbiology and Hygiene, Mainz, Germany; Bhakdi et al., 1985; Bhakdi et al., 1993).

2.4. Immunoblots

Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to Hybond-C extra nitrocellulose membrane (Amersham) using a semi-dry transfer apparatus (Tyler Research Instruments, Edmonton, AB) at 150 mA for 1 h, with materials soaked in 25 mM Tris, 192 mM glycine and 1% (w/v) SDS. Alternatively, samples were resolved on a 1% agarose gel in TBE (Tris/borate/EDTA (ethylenediaminetetraacetic acid)) buffer, pH 7.4, and then transferred to Hybond-P polyvinylidene fluoride (PVDF) membrane (Amersham) using a vacuum blotter (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON) at 5 inches Hg for 90 min. Membranes were blocked in 5% (w/v) skim milk powder (Nestlé Canada Inc., North York, ON) and 0.1% (v/v) Tween-20 in PBS or Tris-buffered saline (TBS), for at least 15 minutes. Antibodies used in immuno-detection are listed in Table 2-1; antibodies were diluted to appropriate concentrations with 0.1% Tween-20 in PBS (Chapter 3 and 4) or TBS (Chapter 5). Membranes were exposed to primary antibodies at 4°C overnight and secondary antibodies at room temperature for 1 h, washing 3 × 10 min with 0.1% Tween-20 in PBS or TBS following each antibody treatment. Blots were developed using enzyme-linked chemiluminescence (ECL, Amersham).

Table 2-1. Antibodies used for Immunoblots

Primary Antibodies				
Antigen[a]	Species[b]	Clone[c]	Working Concentration[d]	Source
human dynamin-1	mouse	Hudy-1	200 ng/mL	Upstate Cell Signaling Solutions, Charlottesville, VA
human grB	mouse	2C5	400 ng/mL	Santa Cruz Biotechnology, Inc., Santa Cruz, CA
Pfn (Chapter 3)	mouse	HuPerf-2d4	1:100	Dr. G. Griffiths (University of Oxford, United Kingdom; Baetz et al., 1995)
Pfn (Chapter 5)	rabbit	----------	1 µg/mL	Torrey Pines Biolabs, Inc., Houston, TX
human grA	mouse	GA6	1 µg/mL	Research Diagnostics, Inc., Flanders, NJ

Horse Radish Peroxidase-linked Secondary Antibodies			
Antigen	Species	Working Concentration	Source
mouse IgG	goat	1:3000	Bio-Rad Laboratories, Hercules, CA
rabbit IgG	goat	1:20000	Biorad

[a] Antigen against which antibody is directed
[b] Species in which antibody is generated
[c] Clone name for monoclonal antibodies
[d] Final concentration of antibody during blot incubation. Where concentrations are unknown, dilution factor is given

2.5. Flow Cytometry Assays

2.5.1. Cell labeling methods

2.5.1.1. Tfn-FITC and grB-A488 uptake assay

Suspended cells were incubated in solutions of 50 μg/mL fluorescein-conjugated transferrin (Tfn-FITC; Invitrogen), or 8 μg/mL grB-A488, for up to 60 min: for binding, 2×10^5 cells were suspended with fluorescent substrate prepared in 10 μL labeling buffer (PBS, 0.1% BSA) at 4°C; for uptake, 8×10^5 cells were suspended with fluorescent substrate prepared in 40 μL DMEM containing 0.1% BSA at 37°C. Uptake reactions were stopped thermally by adding 10 μL of the reaction sample to 190 μL ice-cold labeling buffer. For some samples, cells were pre-incubated in labeling buffer with 10 mM mannose 6-phosphate, or as a control, glucose 6-phosphate (M6P or G6P, respectively, both disodium salt dihydrates) for 20 min at 37°C. During uptake, buffer concentrations were adjusted with water to ensure a final $[Na^+]$ of ~160 mM. Labeled cells were incubated in 10 μL 7-AAD (7-amino-actinomycin D) solution (BD Bioscience) for 10 min at 4°C, then fixed in 2% (w/v) paraformaldehyde in PBS. Flow cytometric analysis was performed to quantify the mean fluorescence intensity (MFI) of 7-AAD-negative cells. The relative MFI was calculated by the following equation: (relative MFI) = (sample MFI) – (unlabeled control MFI).

2.5.1.2. Surface labeling of CI-MPR

Suspended cells (2×10^5) were sequentially labeled in a final volume of 20 μl of labeling buffer, with goat antisera with specificity for the human CI-MPR (diluted 1:100,

received from Dr W. Sly; Nolan et al., 1987), then with R-phycoerythrin-conjugated donkey anti-goat IgG F(ab')$_2$ fragment (diluted to 10 μg/mL; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), each incubation for 20 min at 4°C. After each labeling step, cells were washed twice with labeling buffer. Labeled cells were fixed in 2% paraformaldehyde in PBS, then the MFI was determined by flow cytometry. The relative MFI was calculated by the following equation: (relative MFI) = (sample MFI) – (control labeled only with secondary antibody MFI).

2.5.1.3. Measurements of apoptosis

Caspase activation was monitored by labeling with the monoclonal antibody (mAb) M30 (Roche Diagnostics Canada, Laval, QC) according to the supplier's recommendations, followed by the secondary antibody fluorescein-conjugated AffiniPure donkey anti-mouse IgG (7 μg/mL; Jackson) to specifically detect a caspase-cleavage product of cytokeratin 18 (Leers et al., 1999). DNA fragmentation was monitored by TUNEL labeling (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; Gavrieli et al., 1992) using fluorescein-conjugated dUTP (Roche) as the labeling reagent. For Jurkat cells, the procedure was as described by the supplier; HeLa cells required fixation in ice-cold methanol with 5% (v/v) acetic acid (at least 30 min at –20°C) and washing twice with 0.1% Tween-20 in PBS. Loss of $\Delta\Psi_m$ (mitochondrial inner membrane potential) was determined by adding 100 nM TMRE (tetramethyl-rhodamine, ethyl ester, perchlorate; Invitrogen) to samples in the last 15 min of the killing assay. As a control for loss of mitochondrial $\Delta\Psi$ one sample of each cell type was incubated with 50 μM carbonyl cyanide 3-chlorophenyl hydrazone for 15 min at 37°C. As measures of apoptosis, the percentage of M30- and TUNEL-positive, or TMRE-

negative cells were determined by flow cytometry. The percent specific apoptosis was calculated by the following equation: (% specific apoptosis) = 100% × [(sample % apoptosis) – (untreated sample % apoptosis)]/[100% - (untreated sample % apoptosis)].

2.5.2. Flow cytometry procedure

Flow cytometric analysis was performed on cell samples by examining at least 10,000 events on a FACScan (Becton Dickinson, San Jose, CA) with an excitation wavelength of 488 nm (Heibein et al., 1999). The emission wavelengths of fluorescein and Alexa 488 were detected through an FL1 channel equipped with a 530 nm (20 nm band pass) filter; TMRE and R-phycoerythrin through an FL2 channel equipped with a 580 nm (20 nm band pass) filter; and 7-AAD through an FL3 channel equipped with a 650 nm long pass filter. Where both HeLa-Dyn and CTL were present in a sample, 10,000 gated events were examined, such that the gated region excluded ≥97% of cells in the control of CTL alone. Under these gate conditions, 65-85% of HeLa-Dyn cells were examined.

2.6. Killing conditions

2.6.1. Assay conditions for HeLa targets

2.6.1.1. GrB/AdV and staurosporine

Cell monolayers (~2.5 × 10^5 cells) were covered with 300 ng/mL grB and 500 plaque-forming units per cell AdV, or 2.5 μM staurosporine in RHFM. The

staurosporine samples were matched with DMSO controls. After 2 h incubation at 37°C, cells were lifted with trypsin/EDTA and labeled with M30 mAb.

2.6.1.2. GrB and pfn

Suspended cells (2×10^5) were incubated with 1 μg/ml purified grB and pfn in DMEM with 0.1% BSA and 20 mM HEPES, pH 7.4. The final concentration of pfn was sublytic, as determined by 7-AAD exclusion. To compensate for the addition of sodium chloride present in the pfn stock (or in the control buffer) the sample was adjusted to maintain the concentration of Na^+ at ~160 mM, while maintaining Ca^{2+} at 2 mM. After 2 h at 37°C, samples were labeled with M30 mAb.

2.6.1.3. CTL degranulate material

HighDegran material were obtained fresh one day, and stored at –80 °C overnight until use in a killing assay. Before freezing for storage, the grB activity was estimated against a standard curve for IEPD-pNA cleavage.

To kill, HighDegran was added to cells (2 to 2.5 $\times 10^5$) at an equivalent of 125 to 1000 ng/mL grB activity. For some assays, HighDegran was pre-incubated for 20 min at 37°C with 45 μM grB-specific inhibitor (received from Dr N.A. Thornberry, Merck Research Laboratories, Rahway, NJ; Willoughby et al., 2002) or an equivalent volume of DMSO (0.45% (v/v)), so that upon direct addition to samples, the final concentration was 20 μM inhibitor (or 0.2% DMSO). The final sample (25 μL) was prepared in degranulation media supplemented with 0.1% BSA, 10 mM HEPES pH 7.4, and 2 mM $CaCl_2$. After 3 h at 37°C, samples were labeled with M30 mAb.

2.6.1.4. CTL

Cells (in suspension for TMRE-labeled assays; monolayers for M30- and TUNEL-labeled assays) were incubated with hCTL at approximately 1:1 (effector/target (E/T) ratio) in RHFM supplemented with 2 μg/mL ConA at 37°C, then labeled with their respective apoptotic markers. Some assays were performed in the presence of EGTA (ethyleneglycoltetraacetic acid; 10 mM magnesium chloride, 5 mM EGTA, pH 8.0), or 300 ng/mL neutralizing anti-human Fas mouse monoclonal IgG_1, clone ZB4 (Upstate Cell Signaling Solutions, Charlottesville, VA). Alternatively, hCTL were pre-incubated with 10 nM concanamycin A for 2 h at 37°C.

2.6.2. Assay conditions for Jurkat targets

Cell suspensions (2×10^5) were incubated in DMEM and 0.1% BSA with grB from various sources (purified, isolated granules, hCTL degranulate), with or without additional factors, for 3 h at 37°C. Where indicated, AdV was used at 100 plaque-forming unites per cell; pfn was routinely added at a 10-fold dilution, unless otherwise stated; and SLO was added at 300 ng/mL. Where indicated, 20 mM M6P was added to cells at room temperature for 15 min prior to addition of apoptotic stimuli. Since pfn and granule stocks contain 1 M sodium chloride, and there are extra sodium ions associated with M6P disodium salt, the Na^+ concentration was adjusted as necessary to within 140-160 mM. The Ca^{2+} concentration was maintained at 2 mM.

2.6.3. Assay conditions for lymphoblast and L1210 targets

Two-day ConA-stimulated mouse lymphoblasts and L1210 were treated with alloreactive mouse CTL (5:1 E/T), and DNA fragmentation was assessed by [^{3}H]thymidine release, or cell permeabilization by chromium release, as described previously (Garner et al., 1994). In brief, target cells were labeled with [^{3}H]thymidine for 24 h or ^{51}Cr for 1 h, then challenged with CTL for 2 or 4 h, respectively. [^{3}H]DNA fragments were quantified in the supernatants of cells lysed with 0.5% Triton X-100, and ^{51}Cr was measured in the supernatants of whole cells. Percent specific cell death was calculated by the following equation: (% specific cell death) = 100% × [(sample label release) – (spontaneous label release)]/[(100% label release) – (spontaneous label release)].

2.7. Gel filtration analysis of CTL degranulate

hCTL (2×10^8 in 14 mL Phenol Red-Free RPMI with 2% FCS) were applied to two 100-mL petri dishes that had been coated with 2 µg/mL anti-CD3 (clone HIT3a) overnight at 4°C. Degranulation was allowed to occur over 4 h at 37°C. Degranulate material was collected from the supernatant, then using a 10-kDa cut-off Amicon Ultra 15 centrifugal filter (Fisher), was concentrated at 2800 × *g* for 20 min, re-diluted in 5 mL CHAPS buffer (20 mM Tris, pH 7.4, 137 mM sodium chloride, 1% CHAPS) and concentrated, to a final volume of 200 µL. The concentrated sample was then loaded onto a Superose 6 10/30 gel filtration column (Amersham) hooked-up to an ÄKTA FPLC system (Amersham) with UNICORN software (Amersham), and resolved with CHAPS

buffer at a flow rate of 0.5 mL/min. A_{280} was monitored throughout, and 0.5 mL fractions were collected for analysis of grB activity by IEPD-pNA cleavage.

2.8. Far Western

Samples of either purified grA (200 µg/mL) or purified grB (180 µg/mL) were pretreated with EndoH (0.5 U/mL in 0.05% (w/v) BSA, 50 mM sodium phosphate, pH 5.5), or calf intestine phosphatase (100 U/mL in 0.05% BSA, 10 mM sodium chloride, 5 mM Tris-HCl, pH 7.9, 1 mM magnesium chloride, 0.1 mM dithiothreitol; New England Biolabs Inc., Beverly, MA) with or without high concentration phosphate buffer (64.5 mM sodium phosphate dibasic, 21 mM potassium phosphate monobasic), both overnight at 37°C. Samples were resolved by SDS-PAGE, or by non-denaturing agarose gel, and transferred to nitrocellulose or PVDF membrane, respectively, as described for immunoblots. Membranes were blocked overnight at 4°C in BSA binding buffer (1% BSA, 0.2% Tween-20, 5 mM glycerol 2-phosphate in PBS). In BSA binding buffer, membranes were exposed to 65.5 ng/mL soluble, biotinylated CI-MPR (bCI-MPR; received from Dr P. Lobel, Center for Advanced Biotechnology and Medicine, Piscataway, NJ), then 2 µg/mL Neutravidin-HRP (Pierce Biotechnology Inc., Rockford, IL), each for 1 h at room temperature, with three 10 min washes after each incubation. Blots were developed with ECL.

2.9. bCI-MPR pull-down and immunoprecipitation.

For CI-MPR-mediated pull-down, bCI-MPR was allowed to pre-bind to Neutravidin-Agarose beads (Pierce) at a concentration of 7.5 µg/mL with 0.75% BSA in PBS with a 12.5% (v/v) bead slurry. For immunoprecipitation, biotinylated mouse anti-grA (GA28; Research Diagnostics Inc. (RDI), Flanders, NJ) or biotinylated mouse anti-grB (GB10; RDI) were allowed to pre-bind to NeutrAvidin-agarose beads at a concentration of 12.5 ng/mL with 0.1% BSA in PBS with a 12.5% bead slurry. For both pull-down assays, mock samples lacked bCI-MPR or antibody, and pre-binding was carried-out for 30 min at room temperature with constant agitation. Stocks of purified grA or grB (5 µg/mL in degranulation media with 0.1% BSA), or of fresh HighDegran were added to the bead mixtures, such that the additional volume created a bead slurry of 10%. CI-MPR pull-down samples were allowed to incubate 30 min at room temperature, and immunoprecipitations to incubate overnight at 4°C, both with constant agitation. Beads were washed 5 times with PBS, then eluted directly into 2× SDS-sample buffer, and boiled 10 min prior to SDS-PAGE analysis. For analysis of immunoprecipitated materials, since mouse mAbs were utilized both for the immunoprecipitation and immunoblot, non-reducing sample buffer was chosen to avoid overlap of mAb and grA bands. A sample standard of 5 ng grA or grB, or of 1 µl HighDegran was loaded for comparison.

2.10. Sensitivity-Enhanced Silver Stain

To prepare standard controls for analysis of serglycin, HighDegran (diluted to a final concentration of 50% (v/v)), was treated with or without chondroitinase ABC (0.5 U/mL) in reaction buffer (50 mM Tris-HCl, pH 8.0, 1 M sodium acetate, 0.02% BSA) overnight at 37°C. Samples were resolved on a Tris 4-15% SDS-PAGE gradient gel (Biorad). To stain, the protocol was essentially as described (Min and Cowman, 1986; Ikegami-Kawai and Takahashi, 2002). Briefly, gels were sequentially stained with 0.05% (w/v) Alcian Blue 8GX (electrophoresis grade) in 2% acetic acid, and then with a silver stain kit (Biorad) based on the procedure of Merril et al. (1981), beginning from the oxidation step.

2.11. Digital capture of immunoblot and silver stain images

Immunoblots or silver stained gels were scanned at a resolution of at least 450 dpi, with a Duoscan f40 laser scanner (Agfa-Gevaert NV, Mortsel, Belgium) using Agfa FotoLook 3.6 software (Agfa). Images were processed for display using Adobe Photoshop 5.0 software (Adobe systems incorporated, San Jose, CA).

2.12. Statistical Analyses

Differences in cell-associated fluorescence or target cell death were analyzed using a paired, one-tail Student's t-test. Exceptions include grB-Alexa 488 uptake ± M6P or G6P, which was analyzed using paired one-way ANOVA with a Tukey post-test; and

Jurkat-induced CTL degranulate fractionation, which was analyzed using matched Two-way ANOVA. The latter were performed using GraphPad Prism statistical software.

Chapter 3

The granzyme B-serglycin complex from cytotoxic granules requires dynamin for endocytosis[1]

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[1]A version of this chapter has been published. Kirstin Veugelers, Bruce Motyka, Christine Frantz, Irene Shostak, Tracy Sawchuk, and R. Chris Bleackley, 2004. The granzyme B-serglycin complex from cytotoxic granules requires dynamin for endocytosis. Blood. 103: 3845-3853. © The American Society of Hematology.

3.1. Introduction

Natural killer cells and cytotoxic T lymphocytes (CTL) protect a whole organism against dangerous cells, such as those that are infected with virus or are tumorigenic (Trapani and Smyth, 2002). When natural killers and CTL recognize a target cell, the latter is killed by either of two major pathways: the Fas-Fas ligand (Fas-L) pathway, or directional exocytosis of membrane-bound cytotoxic granules present in the cytoplasm of the killer cell. The granules mediate the demise of the target cell via the enclosed cytolytic molecules, which include, among others, a family of serine proteases called granzymes, and the pore-forming molecule, perforin (pfn; Barry and Bleackley, 2002; Russell and Ley, 2002; Lieberman, 2003). The granzymes, via a pfn-dependent mechanism, induce cell death following translocation across the plasma membrane of target cells. Inside the cell, each granzyme fulfills a critical non-overlapping role to induce apoptosis by cleaving specific subsets of substrates, such as caspases (Darmon et al., 1995; Quan et al., 1996) and Bid (Barry et al., 2000; Heibein et al., 2000; Sutton et al., 2000; Alimonti et al., 2001) by granzyme B (grB), and the SET complex (Fan et al., 2003) by granzyme A. But in order to achieve cleavage of substrates, clearly a critical first step is the uptake of granzymes into the target cell.

The original model of granzyme internalization proposes translocation via a pfn pore in the plasma membrane. However, recent evidence suggests that granzymes are first internalized via endocytosis, then are released into the cytoplasm with the help of pfn by an unknown mechanism. This model is predominantly based on studies performed with grB, which has largely served as the granzyme prototype to date. The first evidence that granzyme uptake occurred by endocytosis was the finding that grB

enters cells autonomously (Froelich et al., 1996; Shi et al., 1997; Pinkoski et al., 1998). Furthermore, grB binds to the cell surface in a concentration-dependent and saturable manner (Froelich et al., 1996), suggesting a receptor-mediated endocytic mechanism. The grB endocytosis model has further developed with the identification of the cation-independent mannose 6-phosphate receptor (CI-MPR) as a receptor for grB (Motyka et al., 2000), however a subsequent study has challenged whether the CI-MPR is critical for grB-mediated killing (Trapani et al., 2003). Both studies demonstrated that the CI-MPR could serve as a receptor for endocytosis of grB, since cells displaying low surface-levels of CI-MPR (CI-MPR$^-$) exhibited reduced grB binding and uptake (Motyka et al., 2000; Trapani et al., 2003). However, the latter study showed that the reduced grB accumulation in CI-MPR$^-$ cells could be compensated by increased incubation time or grB concentration (Trapani et al., 2003), arguing that an alternate non-specific mechanism for grB uptake was sufficient for the induction of apoptosis, which would undermine the relative importance of the CI-MPR.

Further questions concerning grB uptake have been raised due to the finding that granule-secreted grB is bound in a complex to the proteoglycan serglycin (SG; Galvin et al., 1999; Metkar et al., 2002; Raja et al., 2002). Since most *in vitro* studies of grB have used the purified molecule, the following issues must be weighed against such studies. The first concern is whether a bulky complex including SG would allow the interaction of grB and a receptor. Secondly, the grB molecule alone has a positive surface charge (Estébanez-Perpiña et al., 2000; Waugh et al., 2000), but when grB binds SG, since binding is charge-dependent (Matsumoto et al., 1995), the charge must be masked. Therefore the free grB molecule differs from SG-bound grB in that the former might

interact with anything negatively charged on the cell surface, including phospholipid head-groups or glycosaminoglycans. Though this charge-dependent interaction between grB and the cell surface would be non-physiological, especially at relatively high concentrations of purified grB, it could be a sufficient means of grB uptake leading to target cell apoptosis.

To consider another aspect of grB uptake, the model system tested here explores a potential role for dynamin. Dynamin is a focal-point for endocytic studies since it is a major factor in many endocytic events, including those of clathrin-coated pits and caveolae (Conner and Schmid, 2003). Further, a dominant negative mutant (K44A) of dynamin can be overexpressed in cells to effectively block dynamin-dependent endocytic pathways (van der Bliek et al., 1993; Damke et al., 1994). While uptake and apoptosis induced by the purified grB molecule have been assessed in the present study, more importantly, induction of apoptosis by SG-complexed grB has been considered. Results indicated the form of grB dictated the relative dependence on dynamin for endocytosis and induction of apoptosis.

3.2. Results

3.2.1. Regulation of dynamin expression in the HeLa-Dyn system

The model chosen to study dynamin-dependent endocytosis was HeLa cells transfected with the wild type (WT) or the K44A dominant negative (DN) human dynamin-1 cDNA (Damke et al., 1995). Herein, the clones will be referred to as HeLa-DynWT or DN cells, respectively. The expression of the dynamin-1 cDNA was under

the control of a tetracycline (tet)-responsive promoter. As previous characterization has shown (Damke et al., 1994), pre-culturing of HeLa-DynWT or DN in the absence of tet for 48 h induced overexpression of dynamin-1 protein, as detected by immunoblot for dynamin (figure 3-1a).

To monitor the effect of DN dynamin expression on endocytosis, the probe of choice was fluorescently labeled transferrin (Tfn-FITC), a molecule shown to be dependent on dynamin for efficient endocytosis (van der Bliek et al., 1993; Damke et al., 1994). Endocytic progress was monitored quantitatively by flow cytometry in cells incubated at 37°C for up to 60 min (figure 3-1b). Tfn accumulated rapidly in cells not expressing dynamin (WT and DN +tet), and the rate of uptake was unaffected by overexpression of WT dynamin (WT –tet). In contrast, overexpression of DN dynamin (DN -tet) suppressed uptake of Tfn. These findings were supported by visual analysis of cells via confocal laser scanning microscopy (unpublished data). This reflected previous findings that in HeLa-DynWT and DN cell lines, expression from the dynamin cDNA constructs was tightly regulated in a tet-dependent manner, and that specifically DN dynamin induced a block in endocytosis.

3.2.2. Dominant negative dynamin suppresses endocytosis of the grB molecule

To begin, grB uptake was monitored in isolation to determine the effect of DN dynamin on uptake, which, for grB, is an event known to occur autonomously of pfn (Froelich et al., 1996; Shi et al., 1997; Pinkoski et al., 1998). In order to track grB, the purified grB molecule was covalently conjugated to the fluorescent tag Alexa 488 (grB-

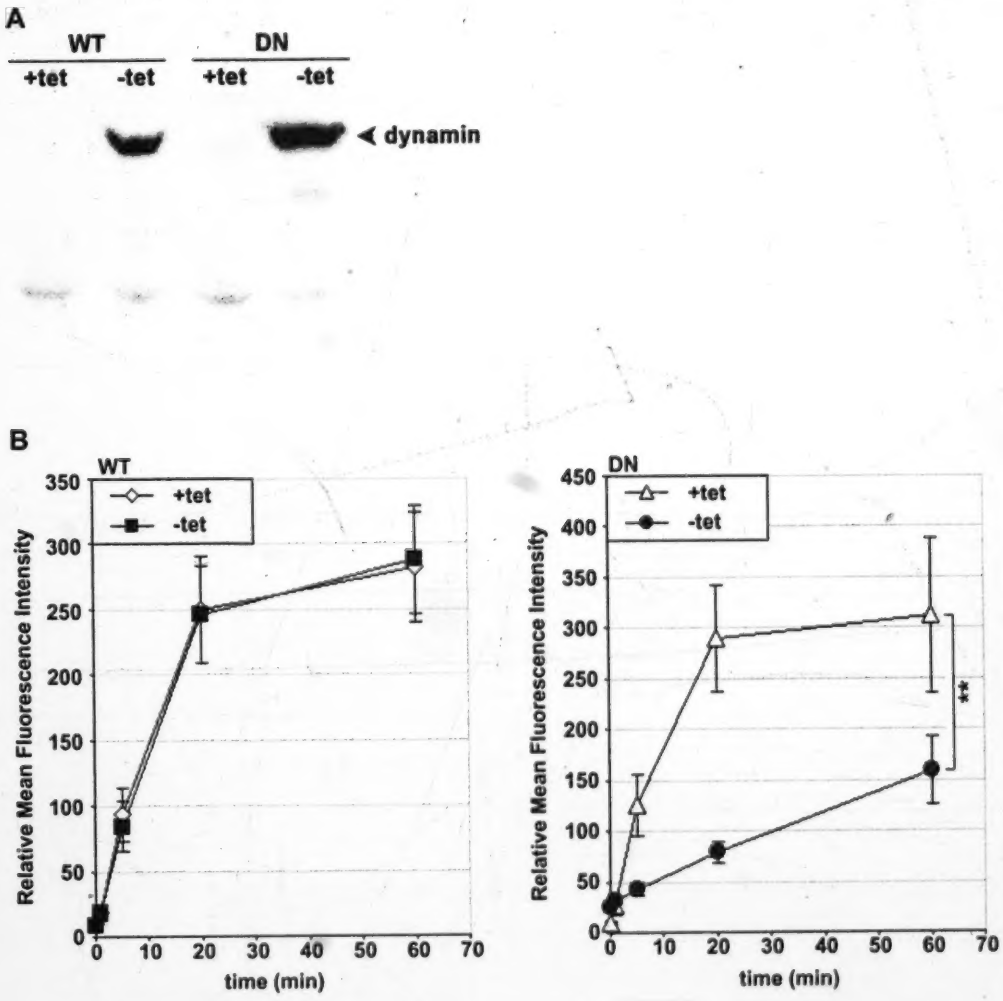

Figure 3-1. Pre-culturing HeLa-DynDN clones in the absence of tet induces over-expression of DN dynamin, and a block in Tfn-FITC endocytosis.
HeLa-DynWT or DN cells were pre-cultured in the presence of tet (+tet) to suppress expression from the transfected dynamin cDNA, or in the absence of tet (-tet) to induce it. *A*, Immunoblot of cell lysates to detect dynamin. *B*, Cells were incubated with Tfn-FITC at 37°C up to one hour. Relative mean fluorescence intensity of viable cells was determined by flow cytometry, to measure total cell-associated fluorescence (surface and internal. The mean ± SD of four independent experiments is shown (**, $0.001 < P < 0.01$).

A488), and uptake was quantified by flow cytometry. Interestingly, a population of cells was observed that were non-viable and acquired high levels of fluorescence when cells were treated with the fluorescently-labeled grB. To address this, cells were labeled with 7-AAD viability probe following the uptake assay, and in the population that did not stain with 7-AAD, uptake of the fluorescently-labeled grB was tracked.

GrB uptake was monitored at 37°C over the course of an hour (figure 3-2a,b). In non-expressing HeLa-Dyn cells (WT and DN +tet) and in cells overexpressing WT dynamin (WT –tet), grB accumulated to significant levels over the period of an hour. In contrast, cells overexpressing DN dynamin (DN –tet) accumulated fluorescence at a significantly slower rate compared to the non-expressing control cells (DN +tet). After 60 min, the DN dynamin-expressing cells showed a ~7-fold accumulation of fluorescence, compared to ~15-fold in the non-expressing control cells, representing a 50% decrease in the rate of grB endocytosis. Thus overexpression of dominant negative dynamin significantly inhibited uptake of the grB molecule. It is apparent, however, that a significant level of grB endocytosis continued in the presence of dominant negative dynamin, as has been shown previously (Trapani et al., 2003).

3.2.3. Dynamin-dependent uptake of the grB molecule occurs via the CI-MPR

Previous studies have indicated that receptor-mediated uptake of the purified grB molecule occurs via the CI-MPR (Motyka et al., 2000; Trapani et al., 2003). Uptake of the purified grB molecule was therefore monitored in response to blocks in endocytosis by CI-MPR and/or dynamin. To interfere with CI-MPR-mediated uptake, receptors were

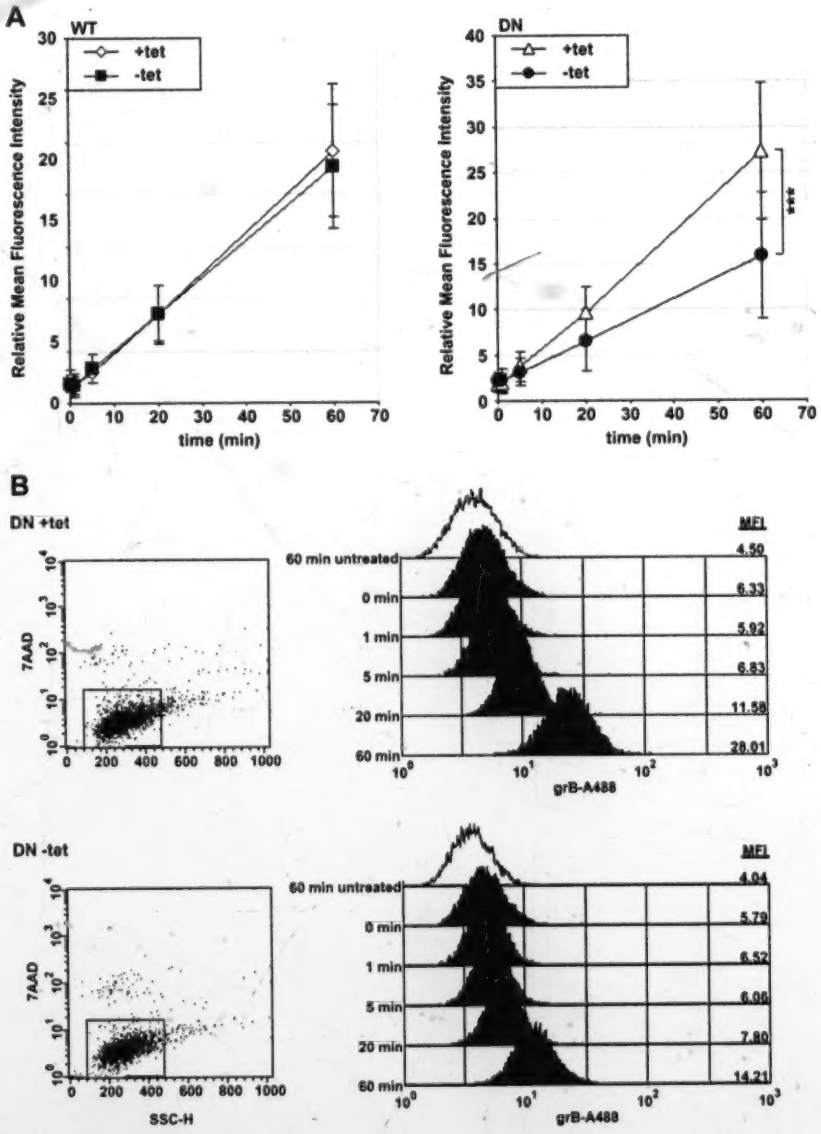

Figure 3-2. Uptake of the purified grB molecule is dependent on dynamin and the CI-MPR.

HeLa-DynWT or DN cells were treated to suppress (+tet) or induce (-tet) overexpression from the transfected dynamin cDNA. The relative mean fluorescence intensity was determined by flow cytometry, to measure total cell-associated fluorescence (surface and internal). *A*, Cells were incubated with grB-A488 at 37°C for up to one hour, and viable cells were analyzed. *B*, Representative flow cytometry data of grB-A488 uptake in HeLa-DynDN cells. The mean fluorescence intensity (MFI) is noted along the right-hand side of the histogram charts for cells, untreated at 60 min, or exposed to grB-A488 from 0 to 60 min. (continued)

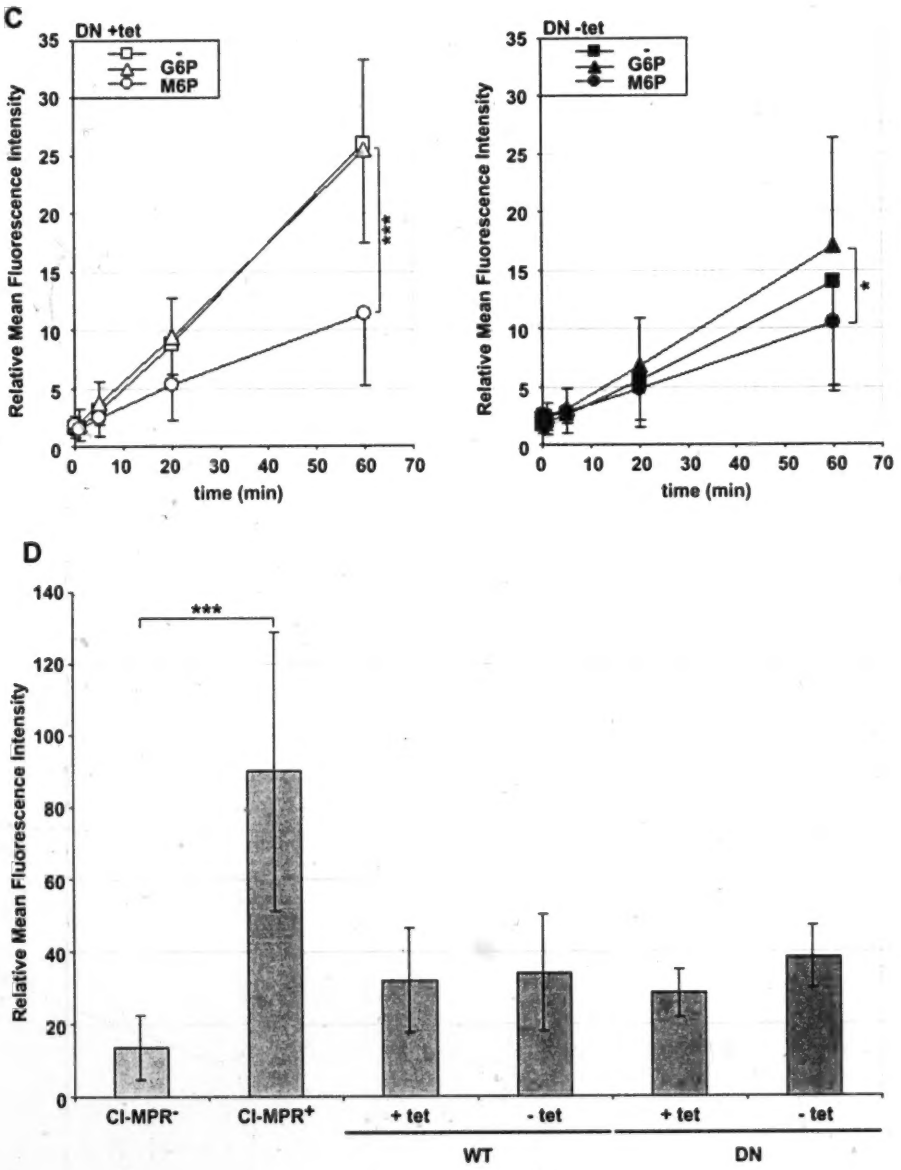

Figure 3-2, continued.

C, HeLa-DynDN cells were incubated with grB-A488 under normal conditions (-), or in the presence of 10 mM G6P or M6P, and viable cells were analyzed. *D*, Cells displaying negligible or high surface levels of the CI-MPR (CI-MPR$^-$ or CI-MPR$^+$, respectively), or HeLa-Dyn cells were surface labeled for CI-MPR. The mean ± SD of four (A) three (C) or five (D) independent experiments are shown (*, $0.01 < P < 0.05$; ***, $P < 0.001$).

saturated with 10 mM mannose 6-phosphate (M6P), or as a control, with glucose 6-phosphate (G6P), then uptake of grB-A488 was monitored (figure 3-2c). Overall, treatment with G6P did not significantly affect the rate of fluorescence accumulation. In contrast, M6P significantly suppressed accumulation of fluorescence in normal cells (+tet). This indicated that uptake of grB was strongly dependent on the CI-MPR. In fact, comparable inhibition by M6P and DN dynamin was observed. Importantly, combining both defects did not have an additive effect with respect to attenuating grB uptake, but rather, the combined block inhibited uptake to a similar degree as using either block independently. This, therefore, suggested that when grB was taken up via the CI-MPR, it also relied on dynamin. This result is not surprising, given that CI-MPR uptake is clathrin-mediated (Willingham et al., 1981). Notably, differential expression of WT or DN dynamin did not alter CI-MPR surface expression (figure 3-2d), therefore the decreased levels of grB uptake in the presence of DN dynamin could not be attributed to changes in CI-MPR surface levels.

3.2.4. Endocytosis-dependent apoptotic stimuli are sensitive to dominant negative dynamin

In order to test whether inhibition of endocytosis would result in attenuation of killing, adenovirus (AdV)-assisted grB-entry was assessed (figure 3-3a), since uptake of AdV itself is dynamin-dependent (Wang et al., 1998). Importantly, though the read-out was apoptosis, which was dependent on the internalization of grB, this step was absolutely dependent on co-internalization of the AdV. Non-expressing HeLa-Dyn cells (WT and DN +tet) or WT dynamin-overexpressing cells (WT –tet) displayed comparable

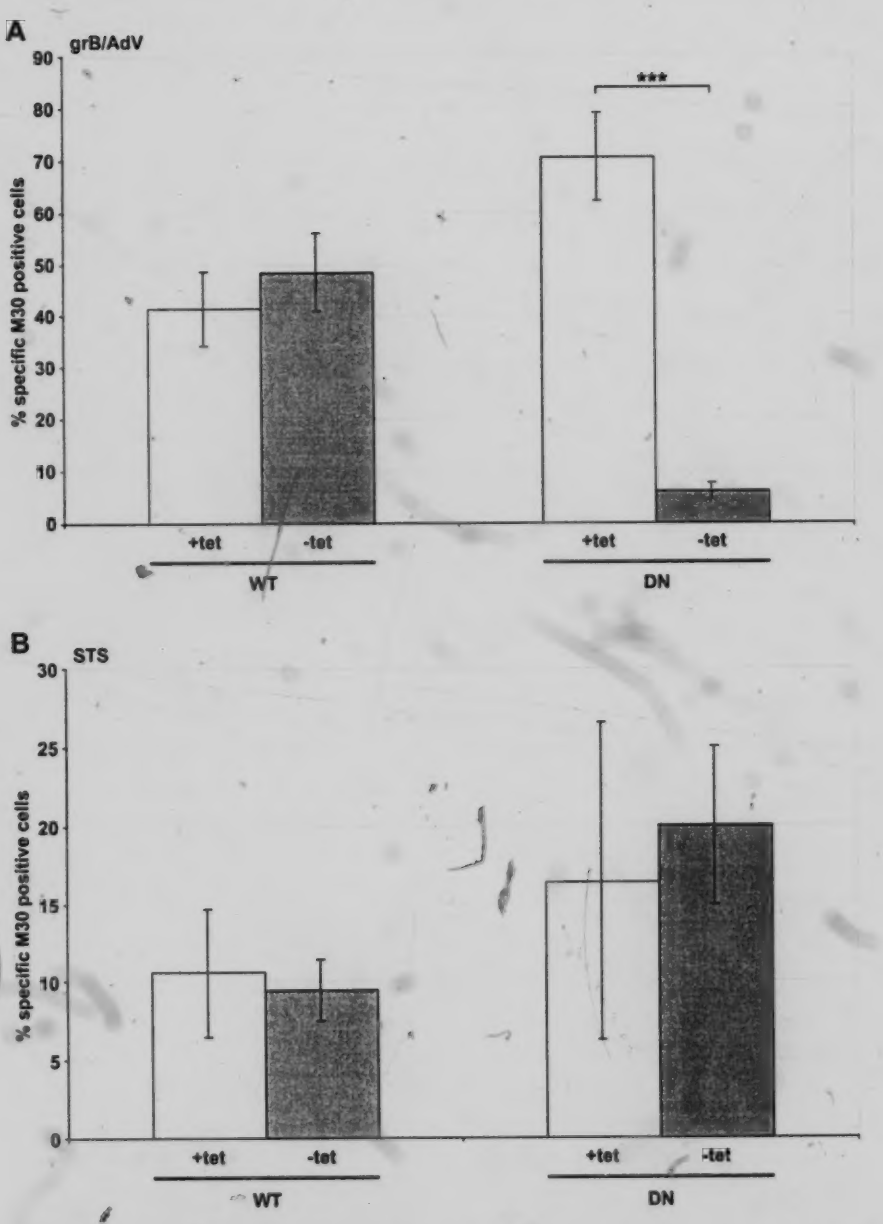

Figure 3-3. Death induced by grB and AdV, but not by staurosporine, is dependent on dynamin.

HeLa-DynWT or DN cells were treated to suppress (+tet) or induce (-tet) overexpression from the transfected dynamin cDNA. Caspase activation was assessed by labeling with M30 mAb. The number of M30-positive cells was quantified by flow cytometry. *A*, Cells were treated with grB and AdV for 2 h at 37°C. *B*, Cells were treated with staurosporine (STS) for 2 h at 37 C. The mean ± SD of four independent experiments are shown (***, $P < 0.001$).

caspase activation, as indicated by labeling with mAb M30. In contrast, in cells overexpressing DN dynamin (DN –tet), M30-labeling was effectively abolished, which indicated AdV had not been effectively internalized. These data demonstrated that an attenuation in cell death would be observed if the apoptotic stimulus was reliant on dynamin for endocytosis.

It was also important to consider whether the endogenous apoptotic machinery would be affected by the overexpression of dynamin or the conditions used to induce overexpression. Cell death induced by staurosporine was therefore monitored to address this question (figure 3-3b). Similar levels of M30 labeling were observed whether cells did (-tet) or did not (+tet) overexpress either form of dynamin. These results suggested that the apoptotic machinery was unaffected, and any observed decreases in apoptosis in the following studies were due to a defect in the uptake of the stimulus, rather than a defect in the apoptotic machinery.

3.2.5. Dominant negative dynamin suppresses apoptosis induced by the purified molecules grB and pfn

Though the evidence suggested that uptake of grB could occur in a dynamin-dependent manner, it was important to determine whether this occurred in the presence of pfn, and, if so, whether such a pathway was important for induction of apoptosis (figure 3-4a,b). Non-expressing HeLa-Dyn cells (WT or DN +tet) were sensitive to treatment with purified grB and pfn, but apoptosis in cells overexpressing DN dynamin (DN –tet) was only half as much as the control. Notably, overexpression of WT dynamin (WT –tet)

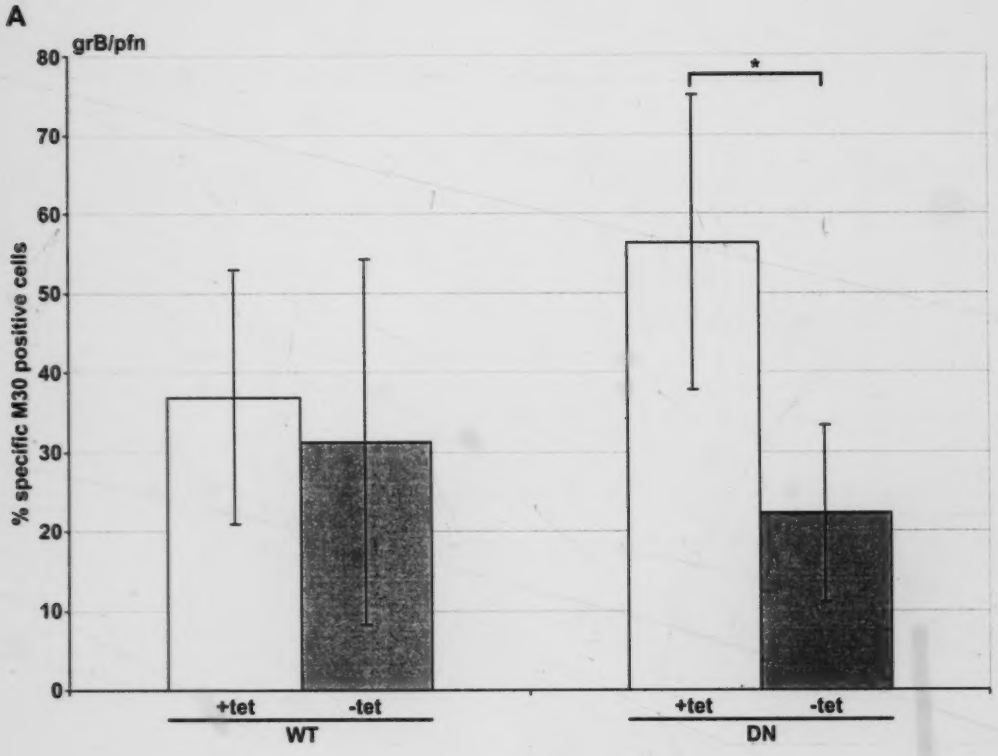

Figure 3-4. Apoptosis induced by the purified molecules grB and pfn is sensitive to dominant negative dynamin.

HeLa-DynWT or DN cells were treated to suppress (+tet) or induce (-tet) overexpression from the transfected dynamin cDNA. Cells were treated with the purified molecules grB (1 mg/mL) and sublytic pfn for 2 h at 37°C. Caspase activation was assessed by labeling with M30 mAb, followed by flow cytometry. *A*, The mean ± SD of three (WT) or four (DN) independent experiments are shown (*, $0.01 < P < 0.05$). (continued)

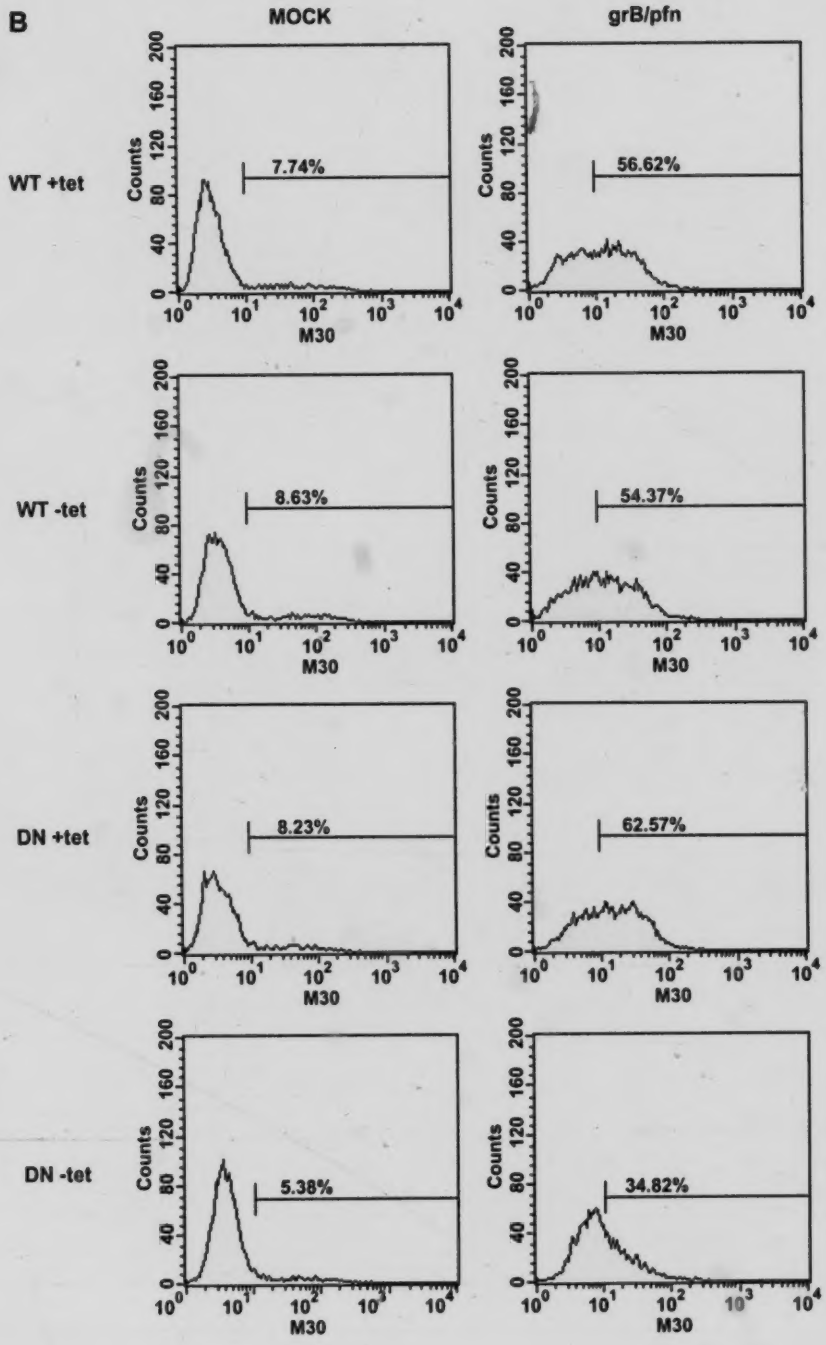

Figure 3-4, continued.
B, Representative flow cytometry data of untreated (mock), or grB and pfn-treated cells.

did not have the same inhibitory effect as DN dynamin. This suggested that overexpression of the DN dynamin limited the ability of grB and pfn to activate the caspase cascade. Presumably DN dynamin interfered with endocytosis of grB, though pfn may or may not have been affected. Interestingly, since killing was only reduced by half in the presence of DN dynamin, these data also implied the existence of dynamin-independent means of grB uptake, which is consistent with the grB-A488 uptake data (figure3-2a,b), as well as previous findings (Trapani et al., 2003).

3.2.6. Apoptosis induced by high molecular weight degranulate material is dynamin-dependent

Mounting evidence has suggested that many molecules are involved in the granule-mediated killing pathway, and, in particular, SG is one such molecule, and is proposed to usher grB (Galvin et al., 1999; Raja et al., 2002) and pfn (Metkar et al., 2002) from the granule to the target cell. To better mimic granule-mediated killing, degranulation of human CTL (hCTL) was induced with an anti-CD3 mAb, then the degranulate material was fractionated over a 100 kDa cut-off centrifugal filter to separate the SG complex, an estimated 250 kDa, from smaller molecular weight material that might include free grB, 32 kDa. To compare and characterize the fractions, they were resolved on a neutral, non-denaturing 1% agarose gel, then blotted and probed for grB (figure 3-5a(i)). Firstly, grB had primarily partitioned into the high molecular weight fraction of degranulate material (HighDegran). Secondly, grB in HighDegran migrated strongly toward the anode, which is consistent with migration of a grB-SG complex (Raja et al., 2002). To further test the nature of the complex, samples were pre-treated with 2

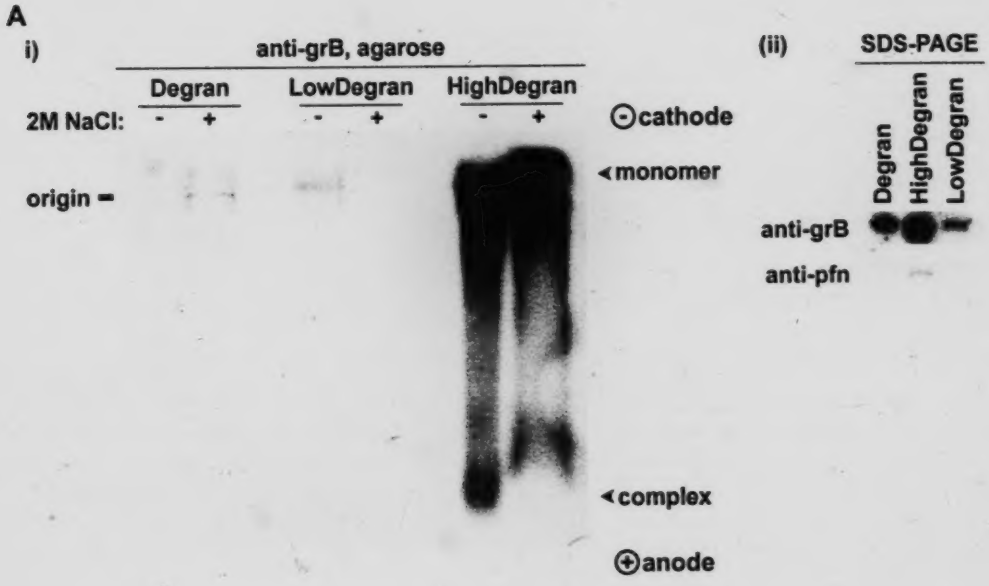

Figure 3-5. SG-bound grB is taken-up predominantly by a dynamin-dependent mechanism.

hCTL were induced to degranulate over immobilized anti-CD3 mAb, and the supernatant was collected and fractionated over a 100 kDa cut-off centrifugal filter. HeLa-DynWT or DN cells were treated to suppress (+tet) or induce (-tet) overexpression from the transfected dynamin cDNA, then incubated for 3 h at 37°C with the >100 kDa fraction of degranulate material (HighDegran). Caspase activation was quantified by labeling with M30 mAb followed by flow cytometry. *A*, Equivalent volumes of hCTL degranulate (Degran), the < 100 kDa degranulate fraction (LowDegran), and HighDegran were resolved on a gel prior to immunoblotting. (i) Samples, pre-incubated ± 2 M sodium chloride, were resolved on a 1 % agarose gel in TBE, pH 7.4, and blot was probed for grB. (ii) Samples were resolved by SDS-PAGE, and the blot was probed for grB or pfn. (continued)

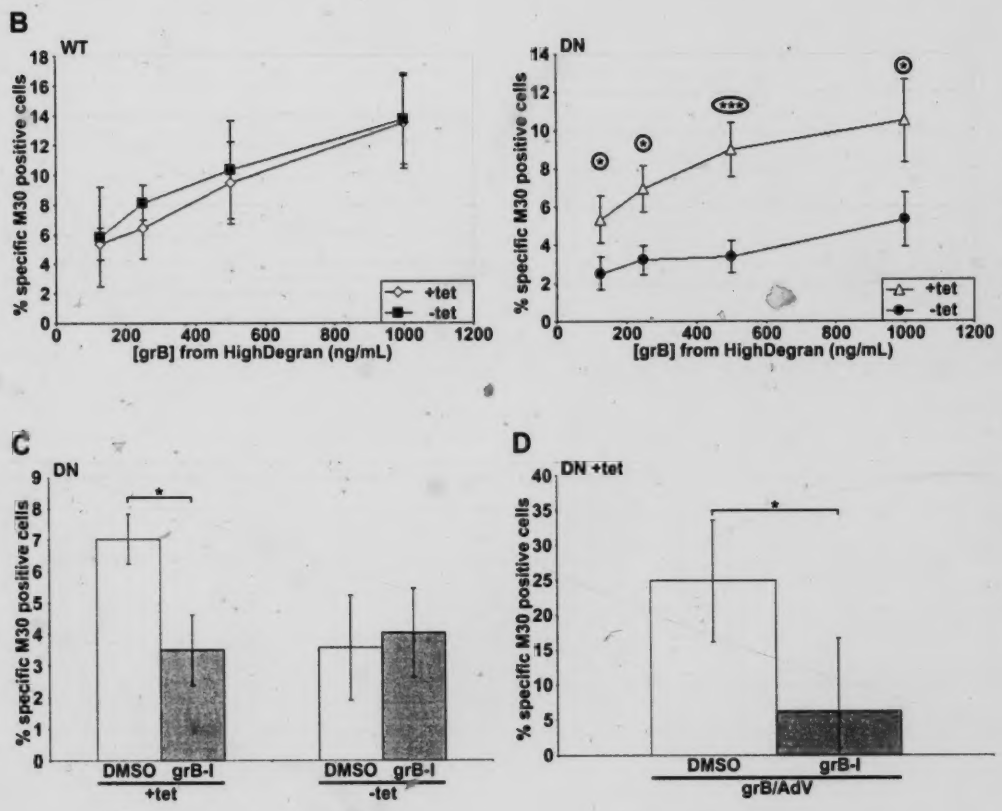

Figure 3-5, continued.
B, Cells were treated with various concentrations of HighDegran, measured in nanograms per milliliter equivalents of grB activity. *C*, HeLa-DynDN cells were treated with HighDegran (500 ng/mL grB activity) that had been pre-treated with DMSO or grB-specific inhibitor (grB-I; 20 mM). *D*, HeLa-DynDN +tet cells were treated with purified grB (500 ng/mL) pre-treated with DMSO or grB-specific inhibitor (grB-I; 20 mM), along with AdV (500 pfu per cell). The mean ± SD of five (B-WT) or six (B-DN) or three (C, D) independent experiments are shown (*, $0.01 < P < 0.05$, ***, $P < 0.001$).

M sodium chloride. This induced dissociation of the complex, though incompletely, and the monomeric, cationic grB migrated toward the cathode. Interestingly, the intensity of the monomeric band was greater than that of the complex, suggesting that the mAb detected the complexed protein less efficiently. To better compare the relative amounts of grB in the various degranulate materials, these were resolved by SDS-PAGE, and an anti-grB immunoblot was performed (figure 3-5a(ii)). The prominent band in the HighDegran fraction confirmed effective partitioning of grB into this fraction.

When cells were treated with the HighDegran alone, significant levels of M30 labeling were detected (figure 3-5b), indicating HighDegran could induce cell death. Remarkably, HighDegran pre-treated with 20 μM of a grB-specific inhibitor (grB-I) poorly induced M30 labeling in cells, compared to the DMSO pre-treated HighDegran (figure 3-5c; +tet). This degree of inhibition served as a measure of grB activity in HighDegran. To test if grB inhibition was quantitative, purified grB was pre-treated with grB-specific inhibitor in parallel, then used to kill cells with AdV (figure 3-5d).

Since grB-mediated killing is dependent on pfn (Shi et al., 1992a; Shi et al., 1992b), detection of grB-mediated killing in HighDegran implied that active pfn also was retained in the HighDegran. Indeed, anti-pfn immunoblot analysis of the degranulate fractions revealed that pfn had effectively partitioned to the HighDegran (figure 3-5a(ii)), likely via binding to SG, as has been previously found (Metkar et al., 2002). Killing by HighDegran could be dramatically enhanced when exogenous pfn was added (unpublished data). However, due to better representation of the *in vivo* granule-mediated killing model, treatment with HighDegran alone was chosen for further studies.

When the role of dynamin in killing by HighDegran was assessed (figure 3-5b), DN dynamin overexpression (DN -tet) consistently suppressed induction of apoptosis compared to the control (DN +tet), though overexpressed WT dynamin (WT –tet) had no effect. It was therefore apparent that induction of cell death by HighDegran was reliant on dynamin-mediated endocytosis. Interestingly, killing by HighDegran was suppressed by overexpression of DN dynamin to a similar extent as by the grB-specific inhibitor. Importantly, treatment with both inhibitors in combination did not further attenuate cell death, but rather was similar to treatment with either inhibitor independently. This implied that the grB in HighDegran was dependent on dynamin for uptake. Since grB in the HighDegran is bound to SG (figure 3-5a(i) and previous studies (Galvin et al., 1999; Metkar et al., 2002)) it was concluded that serglycin-bound grB was critically dependent on dynamin for uptake into target cells.

3.2.7. CTL-mediated apoptosis is dependent on dynamin

A critical test of the model was to challenge the HeLa-Dyn targets with intact CTL. Target cells were mixed with hCTL (1:1 E/T ratio) and various phenotypes of apoptotic induction were assessed. In particular, apoptotic hallmarks known to be mediated by grB were evaluated: activation of caspases (Darmon et al., 1995; Quan et al., 1996) by M30 labeling; loss of mitochondrial inner membrane potential ($\Delta\Psi_m$; Heibein et al., 1999; MacDonald et al., 1999) by loss of TMRE labeling; and oligomerization of DNA (Shi et al., 1992a; Shi et al., 1992b) by TUNEL. Due to the distinct scatter patterns of HeLa-Dyn and hCTL, it was possible to set the gate to exclude > 95% of hCTL from the analyzed cells, and therefore predominantly monitor the HeLa-Dyn population.

Compared to cells not expressing the transfected dynamin gene (WT +tet), the cells overexpressing WT dynamin (-tet) showed equivalent (M30; figure 3-6a) or slightly enhanced (TMRE and TUNEL; figure 3-6b,c) sensitivity to hCTL challenge. In contrast, cells overexpressing DN dynamin (-tet) demonstrated suppressed sensitivity to attack by hCTL, as detected in all three apoptotic assays. These results suggested that CTL-mediated killing was dependent on a dynamin-mediated endocytic mechanism.

Since hCTL not only kill via the granule pathway but are also able to employ the Fas-L to induce apoptosis (Rouvier et al., 1993), the amount of Fas-L-mediated killing was measured by treating samples with EGTA to block degranulation, which is Ca^{2+}-dependent, or by pre-treating hCTL with concanamycin A (CMA) to block pfn-dependent killing (Kataoka et al., 1996). Alternatively, samples were treated with a neutralizing anti-Fas mAb (nαFas) to block Fas-L-mediated killing and measure granule-mediated killing alone. It is worth noting that none of these treatments had appreciable effects on hCTL scatter or labeling profiles. By all apoptotic measurements (figure 3-6a,b,c), EGTA- or CMA-treatment largely abrogated killing, or nαFas had minimal effect, suggesting the hCTL killing observed was pre-dominantly granule-mediated. Together, these results suggested that during granule-mediated killing, some cytolytic factor was dependent on dynamin to mediate its pro-apoptotic effect. The fact that DN dynamin had a suppressive effect on grB-dependent apoptotic characteristics, including caspase activation, loss of $\Delta\Psi_m$, and DNA oligomerization suggested that at least killing through grB is dependent on dynamin-mediated endocytosis.

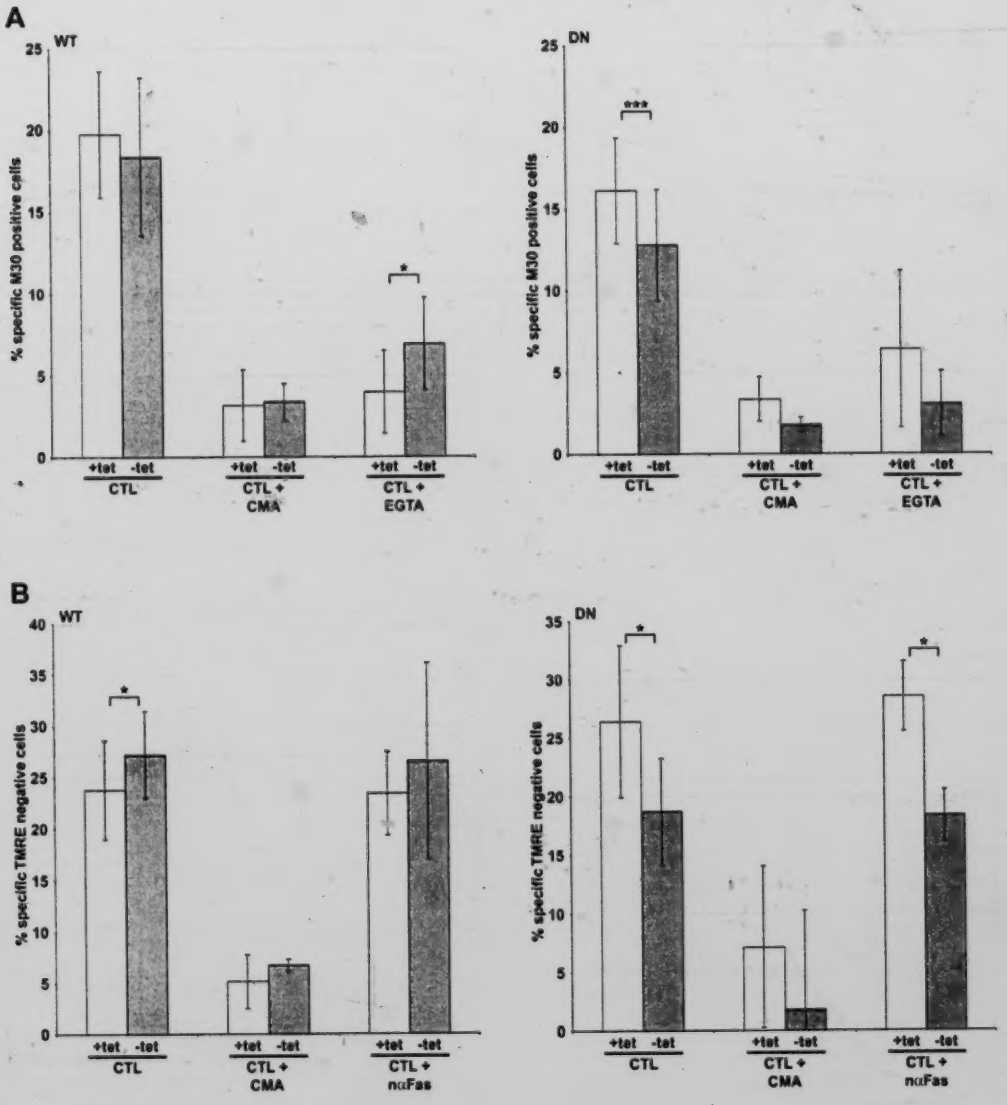

Figure 3-6. CTL-mediated killing is sensitive to dominant negative dynamin. HeLa-DynWT or DN cells were treated to suppress (+tet) or induce (-tet) overexpression from the transfected dynamin cDNA. Cells were treated with CTL (1:1 E/T ratio), with or without EGTA, concanamycin A (CMA) or neutralizing anti-Fas (nαFas). All assays were quantified by flow cytometry. *A*, Caspase activation was assessed after 2 h by labeling with M30 mAb. *B*, Loss of $\Delta\Psi_m$ was assessed after 2 h by monitoring loss of TMRE labeling. (continued)

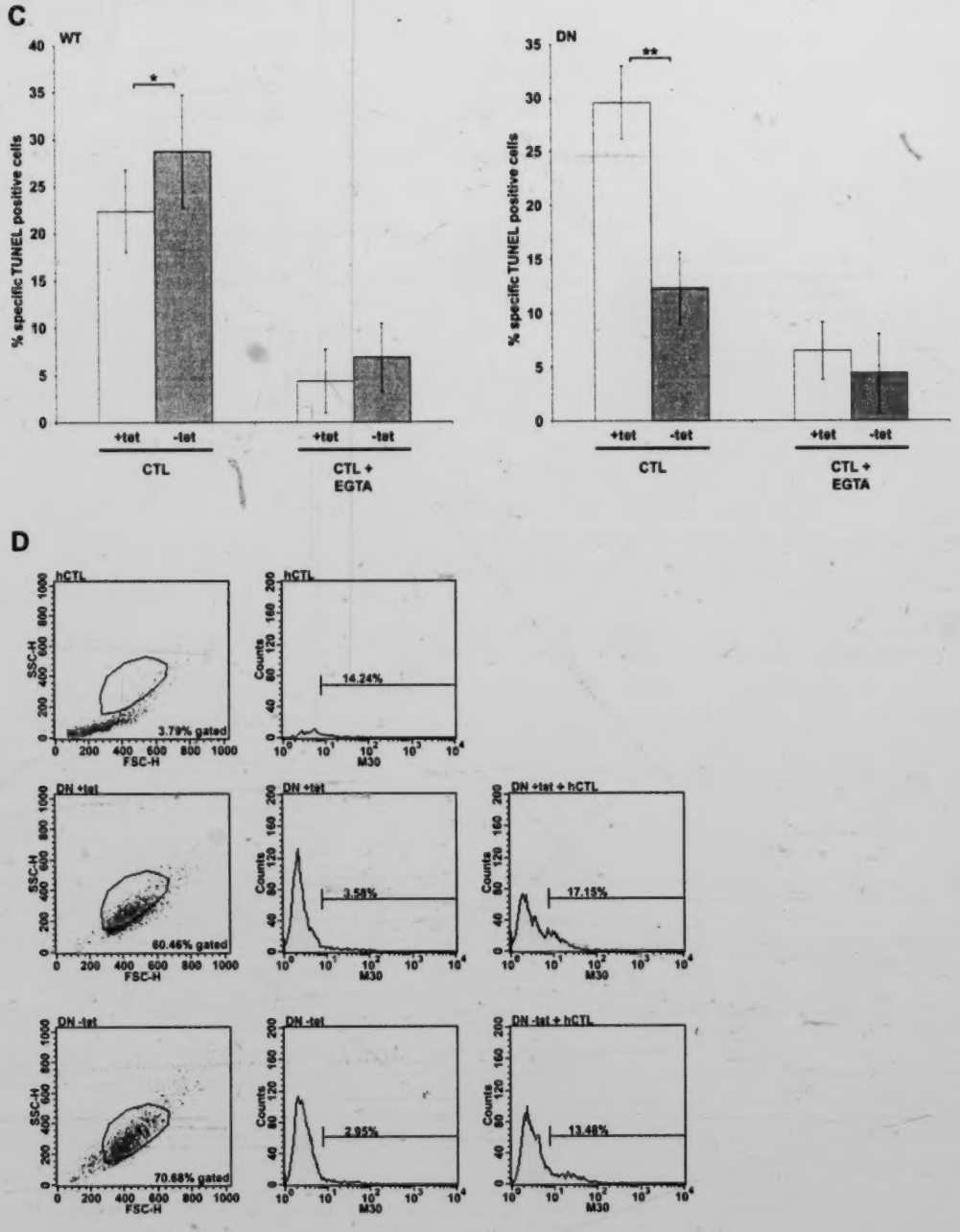

Figure 3-6, continued.

C, DNA oligomerization was assessed after 4 h by TUNEL. *D*, Representative flow cytometry data of hCTL, or HeLa-DynDN ±tet ±hCTL, as monitored by M30 mAb labeling. The mean ± SD of four (A,C) or three (B) independent experiments are shown (*, $0.05 < P < 0.01$; **, $0.001 < P < 0.01$; ***, $P < 0.001$).

3.3. Discussion

The mechanism of cytolytic molecule delivery into target cells during granule-mediated killing has been a topic of debate in recent years. Despite the pervasiveness of a model where uptake of granzymes depends on passage through a perforin pore, mounting studies of grB have suggested uptake of granzymes occurs primarily via an endocytic mechanism, and perforin is involved in the subsequent release of granzymes from the endosomes into the cytoplasm (Barry and Bleackley, 2002; Russell and Ley, 2002; Lieberman, 2003). The data presented in this study have addressed whether dynamin-mediated endocytosis is critical for granule-mediated killing. Importantly, the role of dynamin has not only been considered in the context of a purified component of the granule system, namely grB, but also of the more physiologically relevant degranulate material, as well as of intact CTL. Though the purified grB molecule was only partially dependent on dynamin for endocytosis, grB in HighDegran, found to be SG-bound, was taken-up primarily in a dynamin-dependent manner. In support of this finding, granule-mediated killing by CTL was significantly impaired by overexpression of DN dynamin. Thus during granule-induced target-cell apoptosis, dynamin-mediated endocytosis of grB, and likely other granzymes, is a critical event.

The major caveat of grB endocytic studies to date has been that the primary focus has been the purified grB molecule, while *in vivo* degranulated grB is bound to the proteoglycan SG (Galvin et al., 1999; Metkar et al., 2002). To better address the *in vivo* scenario, CTL degranulate material was collected and fractionated to obtain the high molecular weight SG-bound grB. Killing by HighDegran was sensitive to overexpression of DN dynamin (figure 3-5b). Further, quantification of grB activity in the HighDegran

with a grB-specific inhibitor revealed that apoptosis induced by grB was blocked by DN dynamin (figure 3-5c). Since native agarose analysis of HighDegran suggested the grB was SG-bound (figure 3-5a(i)), these findings suggest that SG-complexed grB is taken up predominantly by a dynamin-dependent mechanism. Since previous reports have indicated that degranulated grB is exclusively SG-bound (Galvin et al., 1999; Metkar et al., 2002), these data imply that *in vivo* the dynamin-dependent endocytic mechanism is the primary means of grB uptake.

In support of a dynamin-dependent endocytosis model for degranulated granzymes, target cell death by granule-mediated CTL-attack was reliant on dynamin (figure 3-6a,b,c). In monitoring caspase activation, loss of $\Delta\Psi_m$ and DNA oligomerization, all three apoptotic markers during granule-mediated killing were suppressed by DN dynamin. The above apoptotic markers are induced by grB (Shi et al., 1992a; Shi et al., 1992b; Darmon et al., 1995; Quan et al., 1996; Heibein et al., 1999; MacDonald et al., 1999), and in particular, caspase activation and DNA oligomerization cannot be effected by other granzymes (Lieberman, 2003). This indirectly supports the model that grB uptake during granule-mediated killing is dynamin-dependent.

It is clear from the results that CTL-mediated killing is both dynamin-dependent and independent. Though grB has been the particular focus of this study, due to the complexity of cytolytic granules, it is probable that factors other than grB are also reliant on dynamin. In particular, all granzymes are likely candidates. In contrast, some cytotoxic molecules must work independently of dynamin, as evidenced by CTL-mediated killing detected even in the presence of DN dynamin.

Though the presented results imply that SG-bound grB is taken-up primarily in a dynamin-dependent manner, in contrast, uptake of free grB has been observed by both dynamin-dependent and -independent mechanisms. In figure 3-2a, while uptake of the purified grB molecule was somewhat suppressed by DN dynamin, significant uptake still occurred. Similarly, apoptosis induced by the purified molecules grB and pfn was only partially sensitive to DN dynamin overexpression (figure 3-4a). In contrast, in a comparable study, evidence suggested a lesser role for dynamin-dependent uptake (Trapani et al., 2003). In this prior study, uptake of purified grB was minimally affected by DN dynamin, and an exclusively dynamin-independent mechanism was detected for cell death induced by purified grB and pfn, as measured by cell permeabilization and clonogenic survival. The contrasts between the previous and the present findings may be attributed in part to differences in assays. The previous study assessed uptake by quantitative confocal laser scanning microscopy, which is less sensitive than quantification by flow cytometry. The cell death assays also are distinct. Arguably, the M30 mAb assay used in the present study is more relevant to the study of grB uptake. To begin, the M30 assay specifically monitors activation of the cytoplasmic caspases, whereas the previous study assessed chromium release, which not only measures apoptosis, but also necrosis, which may be induced by numerous mechanisms. Further, the clonogenic survival assay may not be appropriate for studies where dynamin-dependent endocytosis has been blocked. Consider that even Tfn-FITC, a factor strongly dependent on dynamin for uptake, significantly accumulates in cells when added to cells for up to an hour (figure 3-1b), indicating that the DN dynamin-overexpression system is only relevant for short-term assays. While the clonogenic survival assay is long-term, the

M30 mAb assay monitors immediate apoptotic induction, and so better assesses initial rates of uptake.

Methodology aside, both data sets are similar since they detect dynamin-independent uptake, suggesting an alternate grB-uptake pathway exists. The question remains why the dynamin-dependent grB uptake is detected in some cases and not in others. Accumulating evidence suggests that the dynamin-independent uptake of the purified grB molecule may occur by binding to the cell surface via a charge-dependent mechanism. An important observation is that this alternate uptake pathway has only been detected when uptake of grB has been assessed in the absence of SG. The relevant distinction between free and serglycin-bound grB is that the free molecule has a strong positive charge (Estébanez-Perpiña et al., 2000; Waugh et al., 2000). Importantly, this charge on grB would be masked when complexed to SG, since binding is electrostatically-mediated (Matsumoto et al., 1995). The positive charge of free grB might allow interactions with negatively-charged groups displayed on the cell surface, such as phospholipid headgroups and glycosaminoglycans. While the purified grB molecule could interact with the cell surface via electrostatic interaction, presumably this association would be of relatively low affinity compared to binding to a grB-specific receptor. Consequently, at low concentrations, free grB would first bind to and be internalized by the receptor in a dynamin-dependent manner. At high concentrations, free grB would bind to both the receptor and the cell surface, so that even if receptor-mediated endocytosis were blocked, adsorptive endocytosis would continue to take-up the surface-bound grB. Consistent with this model, the accumulating data suggest that dynamin-independent uptake of purified grB is concentration-dependent. The present

study detected moderate dynamin-independent killing with 1 μg/ml purified grB (figure 3-4), while previous findings demonstrated much stronger dynamin-independent killing when cells were treated, generally, with higher concentrations of purified grB (Trapani et al., 2003).

Previous reports have determined that the receptor for grB is CI-MPR (Motyka et al., 2000; Trapani et al., 2003), an endocytic step that would be dynamin-dependent (Willingham et al., 1981). In support of this, uptake of the purified grB molecule was suppressed by M6P (figure 3-2c). Furthermore, here and in another study (Trapani et al., 2003), when grB uptake occurred in a dynamin-dependent manner, it was also CI-MPR-mediated. The evidence presented here does not validate the direct assumption that, as for free grB, the CI-MPR is crucial for uptake of SG-bound grB. However, a study of CI-MPR involvement in grB uptake strongly implies that *in vivo* the CI-MPR is the primary receptor for uptake of cytolytic granule molecules (Motyka et al., 2000). Strikingly, allograft transplant of CI-MPR$^-$ mouse tumour cells did not lead to rejection of the graft. Though a similar allograft-transplant experiment in a subsequent study led to rejection of the CI-MPR$^-$ graft (Trapani et al., 2003), the relative importance of this data is a matter of further debate, since graft rejection was antibody-mediated, though the interest was in cell-mediated killing. Nonetheless, the *in vivo* receptor(s) for the SG-complexed cytolytic molecules will have important implications for therapies that might benefit from altered interactions between cytolytic molecules and target cells.

In summary, this study has presented evidence to suggest that uptake of the grB-SG complex is critically dependent on dynamin. Furthermore, granule-mediated killing by cytotoxic lymphocytes involves dynamin, arguably for assisting uptake of the SG-

granzyme complex. Importantly, these data also make the distinction that though free grB uptake may occur by both dynamin-dependent and -independent mechanisms, the more physiologically representative SG-bound grB is primarily taken-up in a dynamin-dependent manner. For future studies of granzyme uptake, these findings imply that appropriate selection of materials will be of the utmost importance for elucidating the physiological model of granule-mediated killing.

Chapter 4

The Granzyme B-Serglycin Complex from Cytotoxic Granules Requires a Mannose 6-Phosphate Receptor for Endocytosis[1]

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[1]The following data were generated by Bruce Motyka: Figures 4-1, 4-2b,c, 4-3, and 4-4, as well as supporting data. None of these data have been published previously.

4.1. Introduction

Granzyme B (grB) is an important effector molecule in the granule-mediated killing pathway by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (Lord et al., 2003; Roberts et al., 2003; Trapani and Sutton, 2003). There is intense interest in grB-related mechanisms since killer cell cytotoxicity and thus grB are implicated in both organism homeostasis and pathology, through elimination of virally or oncogenically transformed cells, and through autoimmunity or graft versus host disease, respectively (Barry and Bleackley, 2002; Russell and Ley, 2002; Trapani and Smyth, 2002). GrB, in a perforin (pfn)-dependent manner, activates an apoptotic pathway by cleaving key substrates in the cytoplasm of the attacked cell. Since the substrates are located inside the cell, a critical step is the entry of grB into the target cell.

The predominant hypothesis describing grB uptake into the target cell proposes an endocytic mechanism (Froelich et al., 1996), and there has been considerable discussion surrounding characterization of the pathway. The first receptor model, proposing that the cation-independent mannose 6-phosphate receptor (CI-MPR) is a critical grB receptor (Motyka et al., 2000), has, therefore, been a point of rigorous scrutiny, with much controversy being created by contrasting data. For instance, grB and pneumolysin O (PLO) have been shown to induce apoptosis in a CI-MPR-deficient cell-line (Trapani et al., 2003). Also, using a similar killing system, CI-MPR-dependent killing has been observed at early time-points, but at later times, significant CI-MPR-independent killing was observed (Trapani et al., 2003). These data, therefore, have argued that though a CI-

MPR-mediated grB uptake pathway existed, it might not be critical since another CI-MPR-independent pathway was sufficient to mediate grB uptake leading to apoptosis.

There has been further debate over the importance of the CI-MPR in grB. Using targets derived from CI-MPR knockout mice, CTL effectively induced chromium release and DNA fragmentation irrespective of CI-MPR expression (Dressel et al., 2004a; Dressel et al., 2004b). Because the death responses were blocked by EGTA (Dressel et al., 2004a; Dressel et al., 2004b) and caspase-inhibitors (Dressel et al., 2004a), the CI-MPR-independent killing was attributed to grB. Further, uptake studies using the CI-MPR knockout cell lines demonstrated comparable endocytosis of grB regardless of CI-MPR expression (Dressel et al., 2004a). Again, this raised the contentious matter of a CI-MPR-independent uptake pathway for grB.

The debate has been propelled further by models proposing alternative grB uptake pathways. One study has suggested that heat shock protein 70 (Hsp70) expressed at the cell surface may mediate grB uptake and binding (Gross et al., 2003). Yet another study has attested that grB requires no receptor at all, since a block in grB binding to cell surface heparan sulfate was insufficient to inhibit killing (Kurschus et al., 2005). Importantly, the grB used in the study was recombinant so that it would lack the mannose 6-phosphate (M6P) tag; thus, uptake of grB in the absence of visible binding could not be attributed to the CI-MPR pathway. All in all, these models have contributed to further skepticism over the CI-MPR-mediated uptake of grB.

Additional discussion of the CI-MPR-mediated uptake model has been stimulated by detection of granule-released grB in complex with a large proteoglycan, serglycin (SG; Metkar et al., 2002). Data have suggested that the major form of granule-released

grB was SG-bound (Galvin et al., 1999). In contrast, uptake studies that identified the CI-MPR as the grB receptor had been performed with a purified free form of grB (Motyka et al., 2000). The free grB is small (32 kDa (Poe et al., 1991)) while a grB-SG complex is at least eight times larger (SG alone from the NK-like cell line YT-Indy is ~250 kDa (Raja et al., 2002)), therefore the concern was that complexing grB to SG might sterically hinder, or altogether block binding to the CI-MPR. While this is an important matter, it has not yet been tested.

Binding of grB to SG raises another interesting point regarding grB-receptor interactions. Since the free grB is positively charged, it might efficiently bind to negatively charged cell-surface molecules, such as glycosaminoglycan (GAG) chains or phospholipid headgroups, leading to CI-MPR-independent uptake. In contrast when positively-charged grB binds the big, negatively-charged SG, not only are positive charges on grB masked since this is the basis of the grB-SG interaction (Stevens et al., 1988; Kamada et al., 1989), but also the negative charge on SG would likely have a repulsive effect against the negative charges on the cell surface. As such, complexing to SG would likely direct grB to a more specific receptor, or set of receptors, which may or may not include the CI-MPR.

In studies of grB uptake, including most CI-MPR-directed analyses, the contribution of SG has largely been ignored. For this reason the impact of SG on grB uptake has been examined, with a particular focus on the CI-MPR-mediated uptake pathway. Evidence suggests that SG preferentially directs grB to the CI-MPR for uptake, and that this pathway is critical for grB-induced apoptosis during granule-mediated killing.

4.2. Results

4.2.1. MPR-dependence of grB is affected by conditions of delivery

Recent publications have criticized the model that grB-mediated killing is critically CI-MPR-dependent. Notably, different results were observed in the presence of different functional analogues of pfn, namely adenovirus (AdV) versus PLO. Although killing with grB and AdV was exclusively CI-MPR-dependent, (Motyka et al., 2000), treatment with grB and PLO clearly demonstrated a detectable CI-MPR-independent pathway (Trapani et al., 2003). When grB delivered by AdV and pfn were compared in parallel killing assays, results similar to previous [illegible] were observed. When Jurkat cells were treated with grB and AdV, then a mannose 6-phosphate receptor (MPR)-directed competitive inhibitor, soluble M6P, could block DNA fragmentation (figure 4-1a). However, when Jurkats were treated with grB and pfn, M6P had a minimal impact (figure 4-1b). Thus, grB-mediated killing was dependent on MPRs when delivered by AdV, but not when delivered by pfn. This suggested that though AdV could functionally mimic pfn to deliver grB, it did not do so by an identical mechanism. Further, this highlighted the importance of choosing lytic agents, particularly in uptake studies of granzymes.

Importantly, this was not the complete story. When Jurkat cells were challenged with intact granules, once again the DNA fragmentation response was MPR-dependent (figure 4-1c). Since the cytotoxic granule-induced DNA damage response in Jurkat target cells has been linked to grB activity through caspase activation (Sarin et al., 1997),

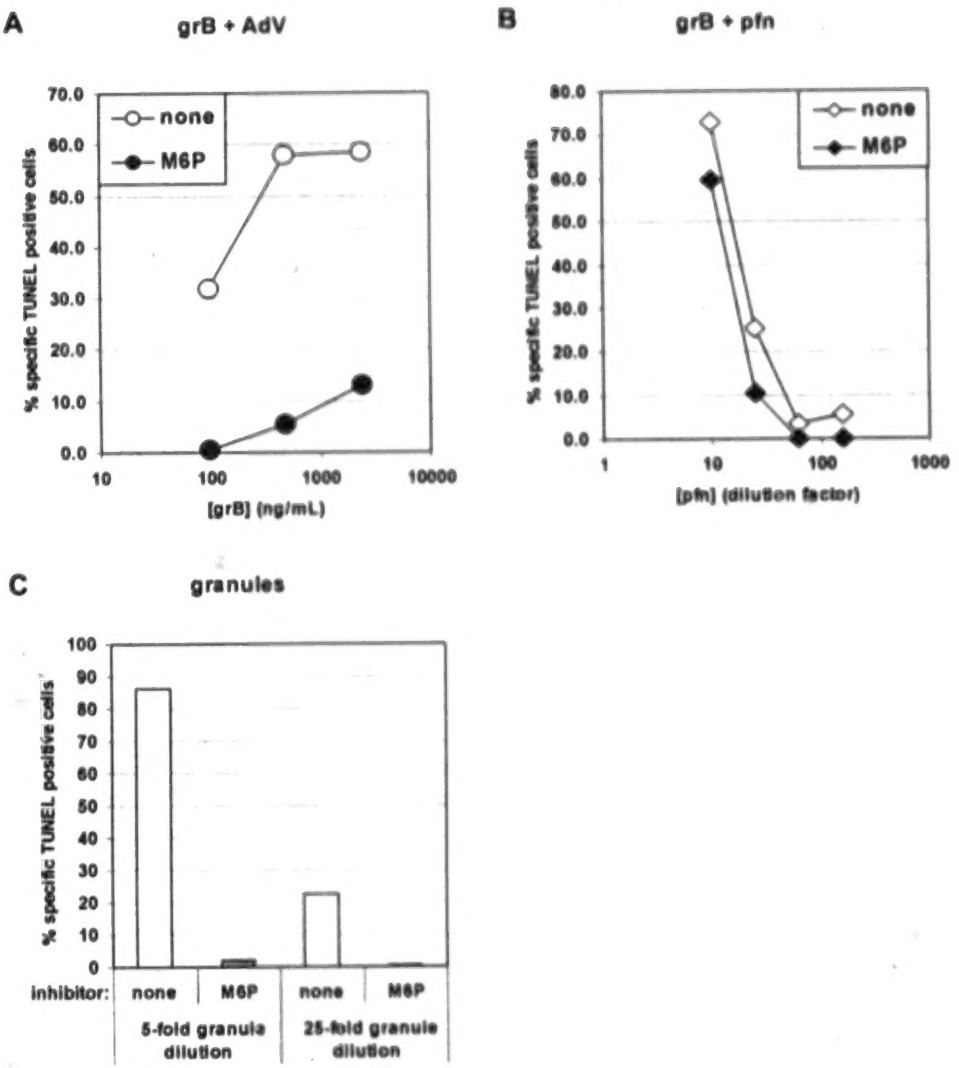

Figure 4-1. GrB and pfn-mediated DNA fragmentation is MPR-independent, but grB and AdV- or granule-mediated DNA fragmentation is MPR-dependent.
Jurkat cells were treated with apoptotic stimuli, in the presence or absence of 20 mM M6P. DNA fragmentation was assessed after 3 h, by TUNEL labeling and flow cytometry analysis. *A*, Cells were treated with the indicated concentrations of grB, along with AdV. *B*, Cells were treated with 500 ng/mL purified grB and the indicated dilutions of purified pfn. The highest concentration of pfn was sublytic, as assessed by exclusion of 7-AAD. *C*, Cells were treated with isolated granules at the indicated dilutions. Data are representative of at least three separate experiments.

this MPR-dependent damage has been attributed to grB activity. Though grB was delivered by pfn as before, killing occurred in the presence of all other granule components, including SG. Given that grB has been found complexed to SG in the granules (Metkar et al., 2002), this seemed a potentially important difference between the grB delivery conditions. Since the negative charge of SG likely would prevent the positively charged grB from binding non-specifically to negative charge on the cell-surface, it seemed likely that a grB-SG complex would preferentially be directed toward a specific receptor. This result suggested a model in which binding to SG would direct grB to a specific receptor, the CI-MPR, while free grB could bind to any negative charge on the cell surface. Further, the data implied that pfn would deliver grB that had been internalized from any binding determinant at the cell surface, while, AdV would only aid in the delivery of grB that had been internalized from an MPR.

4.2.2. GAG-complexed grB is directed to an MPR for pfn-mediated entry

In order to test the hypothesis that SG-complexed grB preferentially relied on the CI-MPR for pfn-assisted apoptosis, an artificial *in vitro* system was developed. Purified grB was incubated with one of two GAGs, either heparin or heparan sulfate (both will be denoted HS in general discussion; figure 4-2a) in order to form a complex. The grB-HS material was then used in killing assays along with pfn to assess the CI-MPR-dependence of grB when its positive charges were blocked by the bound GAGs.

The formation of grB-SG complexes was monitored after grB was incubated either alone or in the presence of heparin. Samples were fractionated over a 100-kDa cut-

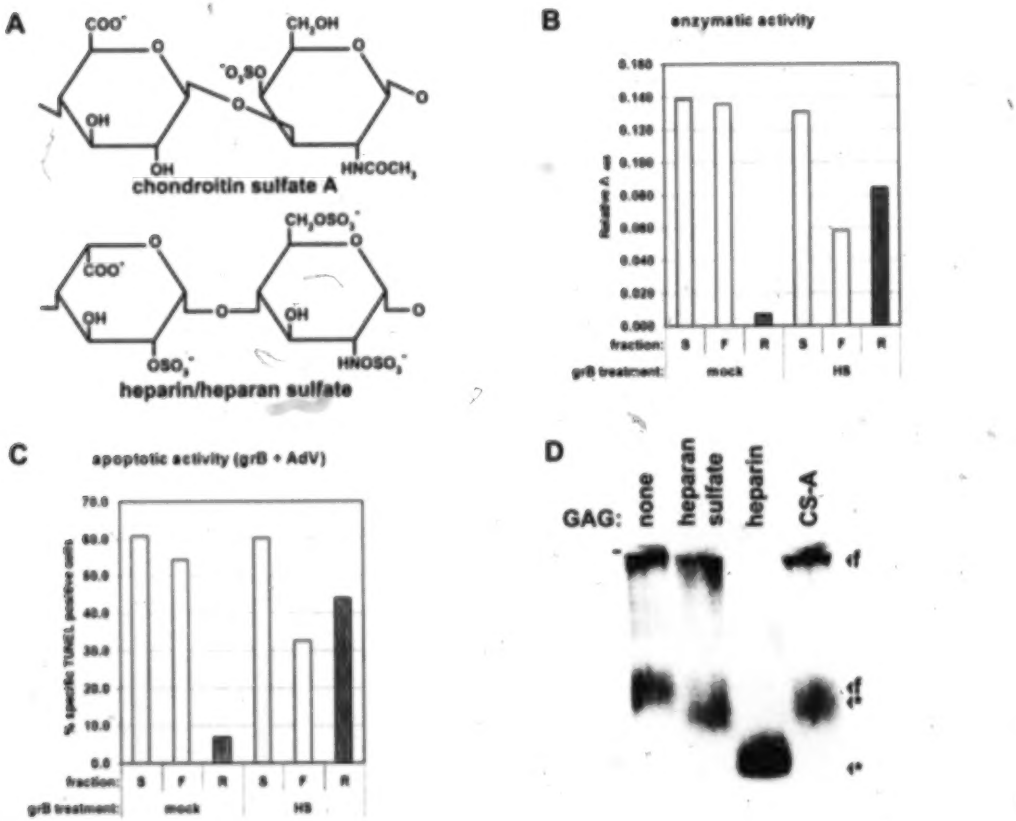

Figure 4-2. GrB complexes to heparan sulfate or heparin as a result of co-incubation.

A, chemical structures of chondrotin sulfate A and heparin/heparan sulfate. Heparin is the most densely sulfated glycosaminoglycan, while heparan sulfate includes variations with fewer sulfate groups (Kolset et al., 2004). *B,C*, Purified grB (20 μg/mL) was mock-treated with PBS, or treated with heparin (100 μg/mL) for a 60 min incubation in media at 37°C. Samples were then fractionated over a 100-kDa cut-off centrifugal filter. Final fractions were re-adjusted back to the original volume with media. *B*, GrB enzymatic activity was assessed in pre-complexed samples (S) and in fractions (F, filtrate; R, retentate) by cleavage of the colorimetric substrate BAADT. The shown relative absorbance readings were within the linear range of the assay, as determined by a standard curve. *C*, GrB pro-apoptotic activity was assessed in a killing assay. Jurkat cells were treated with a 200-fold dilution of pre-complexed sample or fraction in the presence of AdV. DNA fragmentation was assessed after 3 h, by TUNEL labeling and flow cytometry. *D*, Purfied grB (25 μg/mL) was incubated with PBS (none) heparan sulfate (125 μg/mL), heparin (100 μg/mL), or chondroitin sulfate A (CS-A, 50 μg/mL) in media for 90 min at 37°C. Samples were resolved on a non-denaturing agarose gel, and immunoblotted for grB. f, free grB; *, complexed grB. Data are representative of four separate experiments.

off centrifugal filter to separate complexed grB from free grB. Notably, a 50-kDa cut-off filter was ineffective to achieve this separation because free grB was effectively retained by the filter (data not shown). The retentate and filtrate materials were recovered and volumes were re-adjusted to that of the starting material. Then grB activity was detected by cleavage of a colorimetric substrate (figure 4-2b) or in an apoptotic assay with AdV, to induce DNA fragmentation in Jurkat targets (figure 4-2c). By both assays, the mock-treated grB was almost exclusively in the filtrate fraction. In contrast, grB that had been co-incubated with heparin was partially retained on the filter. Similar results were observed when grB was co-incubated with heparan sulfate (data not shown). Thus, treatment of grB with HS led to formation of high molecular weight grB-containing complexes.

As a further test of grB-HS complex formation, materials were analyzed in a gel-shift assay, with grB detected by immunoblot analysis (figure 4-2d). The mock-treated grB had a band near the origin, which is consistent with migration of the positively charged molecule toward the cathode at the top of the gel. It was therefore unexpected to also observe a band that migrated strongly toward the anode. Though this band has not been characterized, it has been speculated that it might represent aggregated grB material. Regardless, when grB was incubated with chondroitin sulfate A (CS-A) as a control (Galvin et al., 1999), a shift in band mobility toward the anode was observed, suggesting the negative charge of the CS-A had induced this shift in grB migration. Similarly, co-incubation of grB with heparan sulfate or heparin resulted in a band shift toward the anode, suggesting that at least a portion of the grB had formed a stable complex with either GAG.

Next, the grB-HS complexes were evaluated for their dependence on the CI-MPR in pfn-assisted killing assays. First, in a control experiment, grB and AdV induced DNA-fragmentation in Jurkat targets whether or not grB was complexed to heparan sulfate, and this killing was always competed by M6P (figure 4-3a). Similar results have been observed when grB was heparin-bound (data not shown). These data indicated that binding of HS to grB did not have a deleterious effect with respect to the pro-apoptotic activity of grB. This was important because when heparan sulfate- (figure 4-3b) or heparin-bound (data not shown) grB was added with pfn to Jurkat targets, now the presence of HS caused a marked reduction in the apoptotic response. This was consistent with the fact that GAGs have been found to inhibit the membranolytic activity of pfn (Tschopp and Masson, 1987). The remaining apoptotic activity of HS-bound grB and pfn was examined in the presence and absence of M6P (figure 4-3c). The results did in fact show that while pfn-mediated delivery of free grB remained MPR-independent, delivery of heparan-sulfate- (figure 4-3c) or heparin-complexed (data not shown) grB was critically MPR dependent.

In a similar set of experiments, SLO was used in place of pfn. Compared to grB and pfn-mediated killing, grB and SLO-mediated killing was even more drastically inhibited by HS (figure 4-3d). Since grB and SLO attack retained no residual pro-apoptotic activity in the presence of HS, dependence on the MPRs could not be assessed. But notably, delivery of free grB by SLO did not require the MPRs, similar to delivery by pfn, suggesting SLO, in contrast to AdV, delivers grB in a manner more like pfn.

Ultimately, the data support the hypothesis that a free grB molecule may bind to various negatively charged sites on the cell surface, be internalized, and be effectively

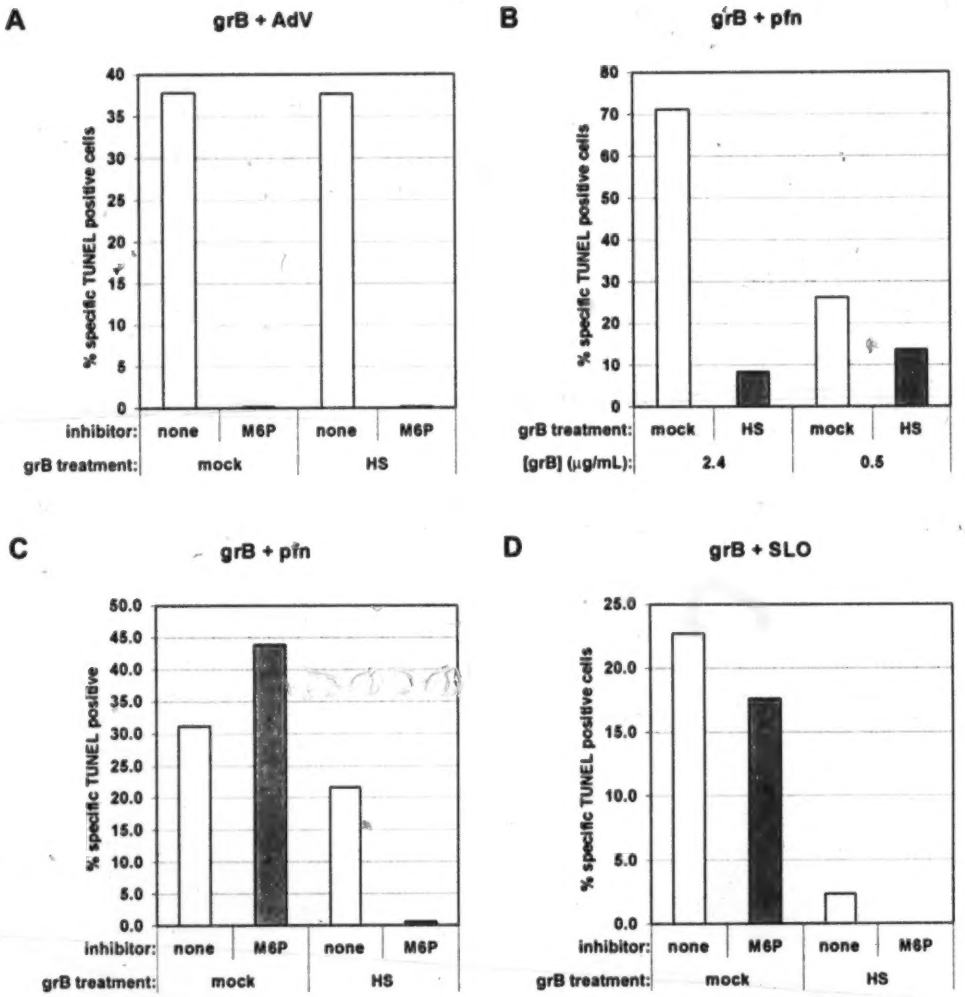

Figure 4-3. GrB-heparan sulfate complexes rely on MPRs to induce apoptosis in the presence of pfn.

Jurkat cells were treated with apoptotic stimuli and inhibitors at 37°C. DNA fragmentation was assessed after 3 h, by TUNEL labeling and flow cytometry analysis. *A*, grB (500 ng/mL) was pre-incubated for 1 h at 37°C in the presence or absence of heparan sulfate (HS, 250 µg/mL), then was added to cells (diluted ~4-fold) along with AdV in the presence or absence of 20 mM M6P. *B*, grB was pre-incubated for 1h at 37°C in the presence or absence of heparan sulfate (HS, 250 µg/mL), was added to cells at the indicated concentrations (diluted ~4-fold), along with pfn (diluted 10-fold). *C*, grB (2.4 µg/mL) was pre-incubated for 1h at 37°C in the presence or absence of heparan sulfate (HS, 250 µg/mL), then was added to cells (diluted ~4-fold) along with pfn (diluted 10-fold) in the presence or absence of 20 mM M6P. *D*, grB (2.4 µg/mL) was pre-incubated for 1h at 37°C in the presence or absence of heparin (100 µg/mL), was added to cells (diluted ~4-fold) along with SLO in the presence or absence of 20 mM M6P. Data are representative of three separate experiments.

delivered by pfn, or even the functional analogue SLO. If, on the other hand, the positive charges of grB are masked, as occurs in the presence of HS or SG, then grB may be preferentially directed to the CI-MPR for uptake.

4.2.3. Apoptosis induced by grB-SG and SLO is MPR-dependent

Given that HS chains are distinct from SG, both with respect to GAG content and the structural contrast of free GAG chains versus intact proteoglycan, it was critical to test whether the observations for HS-bound grB would also be true for SG-bound grB. An important distinction between HS and SG is the overall size, because compared to HS, a large SG molecule would be more likely to sterically block grB from binding to any given receptor, including the CI-MPR.

In order to obtain SG-complexed grB, human CTL (hCTL) were stimulated to degranulate with an immobilized anti-CD3 antibody, and the cell supernatant was collected. As has been shown previously (Galvin et al., 1999; see also chapter 3), under these conditions, grB is released into the supernatant, largely in a SG-complexed form. To compare the pro-apoptotic activity of grB in free and SG-bound forms, apoptosis was induced using purified grB and hCTL degranulate, respectively, along with AdV as the delivery agent. Notably, DNA fragmentation was assumed specifically to indicate the activity of grB in the degranulate, based on observations that this response in Jurkat targets is exclusively grB-mediated (Sarin et al., 1997). Under these conditions, similar pro-apoptotic activity was observed from both purified and degranulated grB (figure 4-4a). When supernatants were taken from hCTL that had not been stimulated with anti-

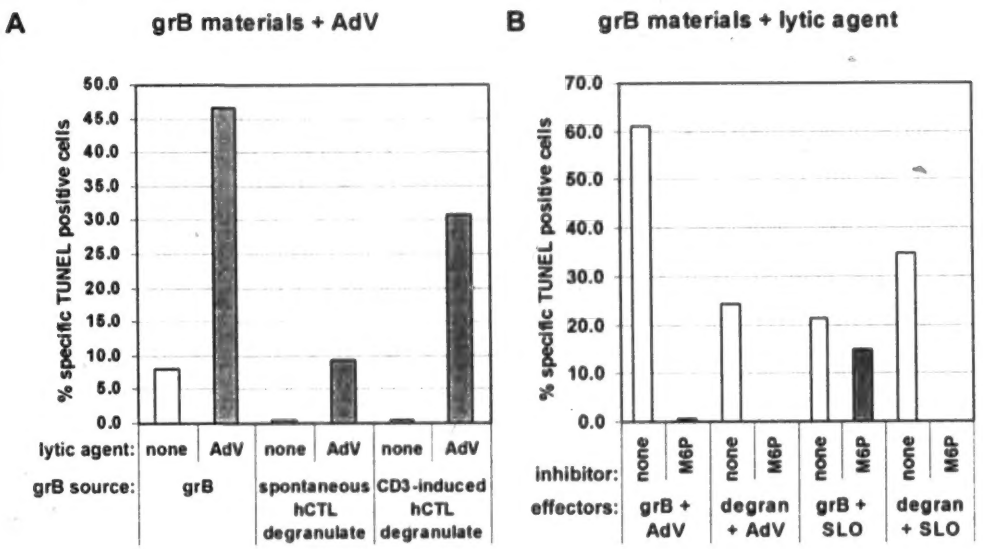

Figure 4-4. Killing by SG-bound grB and SLO is MPR-dependent.
hCTL degranulate material was obtained as the supernatant of a hCTL culture, incubated for 4 h at 37°C in the presence (or absence) of immobilized anti-CD3 antibody. Jurkat cells were treated with apoptotic stimuli and inhibitor at 37°C. DNA fragmentation was assessed after 3 h, by TUNEL labeling and flow cytometry analysis. *A*, grB, either from purified sources (100 ng/mL) or from hCTL supernatants (untreated, ~25 ng/mL; anti-CD3-treated, ~400 ng/mL), was added to cells with or without AdV. *B*, grB, either from purified sources (100 ng/mL) or from supernatants of anti-CD3-stimulated hCTL (~100 ng/mL), was added to cells along with AdV or SLO, in the presence or absence of 20 mM M6P.

CD3, very little pro-apoptotic activity was detected, suggesting degranulation was specifically dependent on CD3 stimulation. It should also be noted that in the absence of AdV, all sources of grB induced negligible levels of apoptosis.

Finally, a similar assay monitored apoptotic responses, using either AdV or SLO as pfn analogues, with a specific interest in MPR dependence (figure 4-4b). Use of AdV helped gauge the relative pro-apoptotic potential of either the pure, free grB or the SG-grB in CTL degranulate. Consistently, this form of killing was always MPR-dependent. In the presence of SLO, both the purified grB and SG-bound grB were able to induce DNA fragmentation. Strikingly, while M6P had no effect on SLO-assisted delivery of the purified grB, it efficiently blocked DNA fragmentation in response to SLO and degranulate material (figure 4-4b). This suggested that the uptake pathway of grB-SG is exclusively via MPRs, most likely the CI-MPR (Motyka et al., 2000). Since there is evidence to suggest that the major granule-released form of grB is SG-bound (Galvin et al., 1999), this implies that the CI-MPR would in fact be critically involved in grB uptake under physiological conditions.

It is rather remarkable that grB and SLO-mediated killing was observed when grB was SG-bound, especially given that HS had an inhibitory effect on SLO activity. However, in the preparation of HS-grB complexes, HS was added in excess, leaving free HS to bind and inhibit SLO or pfn. In degranulate, SG likely is not present in such excess, but rather would be effectively engaged in interactions with the degranulated cargo, such as grB (Galvin et al., 1999) and grA (Masson et al., 1990).

4.2.4. Alloreactive CTL induce granzyme A- and B-independent death in lymphoblast targets

The data presented thus far argue compellingly in favor of CI-MPR-mediated uptake of grB, and even imply a mechanism for the detected CI-MPR-independent uptake pathway (Trapani et al., 2003). Even so, these data are in strong disagreement with CTL killing assays in CI-MPR knockout cell lines. In concanavalin A (ConA)-stimulated lymphoblasts (Dressel et al., 2004a) and in immortalized fibroblasts (Dressel et al., 2004b), CTL-induced DNA fragmentation was comparable between CI-MPR-deficient and wild type cells. Since the induction of DNA fragmentation was sensitive to treatment with EGTA (Dressel et al., 2004a; Dressel et al., 2004b) or caspase inhibitors (Dressel et al., 2004a), it was concluded that this killing was grB-mediated, and did not critically rely on the CI-MPR. Notably, these approaches did not conclusively rule-out a Fas-mediated killing mechanism. Fas-mediated killing is indeed dependent on caspases (Enari et al., 1995; Los et al., 1995; Tewari and Dixit, 1995). Also, though activation of the Fas pathway is calcium-independent (Rouvier et al., 1993), Fas-mediated killing by CTL is still likely EGTA-sensitive, because Fas-ligand localizes to cytotoxic granules, and its surface expression is regulated by the calcium-dependent granule-release event (Bossi and Griffiths, 1999).

The observed CI-MPR-independent killing mechanism of CTL was characterized due to the possibility that it was not grB-mediated. Two-day ConA-stimulated lymphoblasts were challenged with alloreactive CTL derived from $grB^{+/+}$, $grB^{-/-}$, granzyme A $(grA)^{-/-}/grB^{-/-}$, or $pfn^{-/-}$ mice, in the presence or absence of EGTA, and tritium- and chromium-release responses were monitored (figure 4-5a,b). As controls,

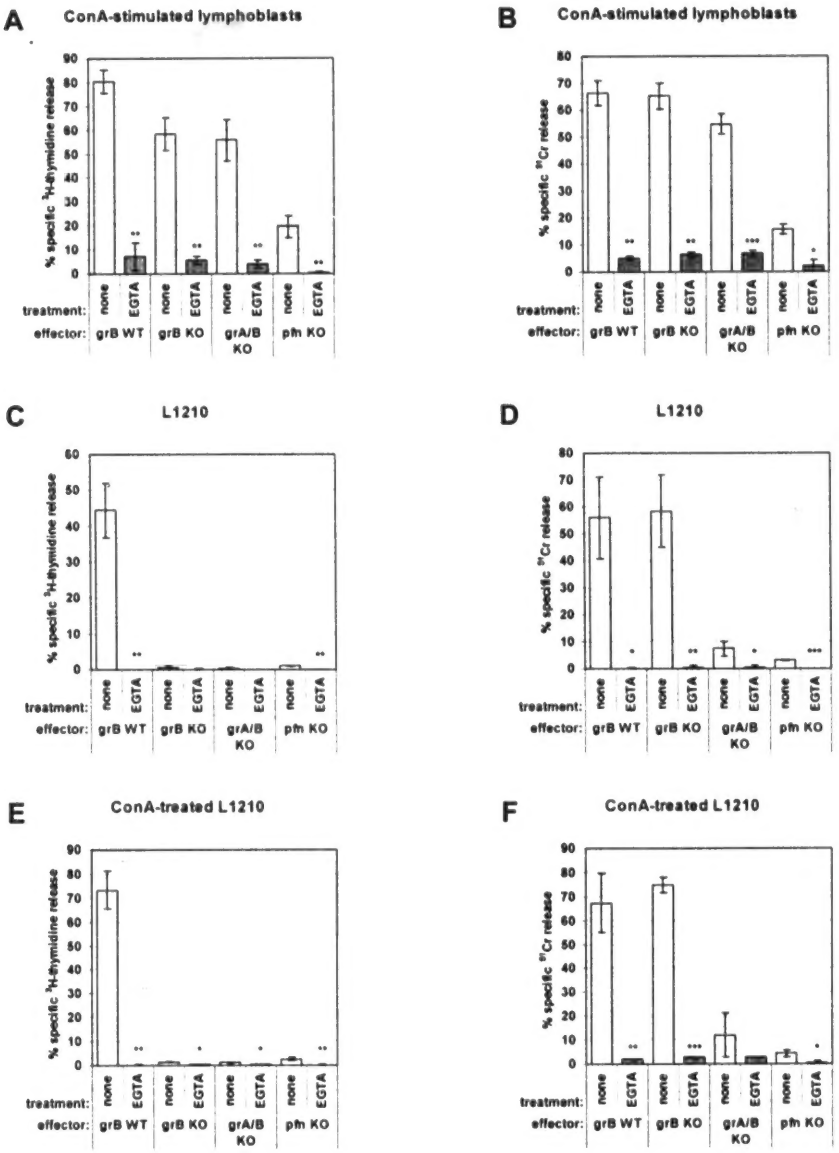

Figure 4-5. Allogeneic CTL attack induces grB-independent DNA fragmentation in ConA-stimulated lymphoblast targets.

2-day ConA-stimulated lymphoblasts (A,B), L1210 (C,D), or 24 h ConA-treated L1210 (E,F) were challenged with alloreactive CTL (5:1 E/T ratio). ConA-treated targets were washed extensively prior to CTL attack. DNA fragmentation in targets was monitored by tritium-release at 2 h (A,C,E), or cell permeabilization by chromium-release at 4 h (B,D,F). KO, knockout; WT, wild type. Data represent the mean ± SD of three separate experiments (*, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$).

L1210, either untreated (figure 4-5c,d) or treated with ConA (figure 4-5e,f), were similarly challenged and monitored. Strikingly, neither DNA fragmentation (figure 4-5a) nor cell permeabilization (figure 4-5b) responses in ConA-stimulated lymphoblasts required grA and/or grB. Further, the death responses were only partially dependent on pfn. Even so, CTL-induced killing was effectively blocked by EGTA (figure 4-5a,b). Most notably, EGTA inhibited the pfn-independent component of killing, which may be mediated by either the Fas pathway or other pfn-independent cytotoxic granule factors. Thus EGTA is not an effective means of distinguishing granzyme and pfn-activated pathways from alternate killing mechanisms in these targets.

This finding cannot simply be attributed to abnormal behaviour of the effector cells. In L1210 a normal response was observed (Simon et al., 1997), because DNA fragmentation was specifically grB- and pfn-dependent (figure 4-5c), while cell permeabilization was dependent on either grA or grB in cooperation with pfn (figure 4-5d). The findings also cannot be attributed to ConA treatment, since the L1210 response was similar when cells were pre-incubated in both the presence and absence of ConA.

While a similar study has not been performed to address the killing events in immortalized fibroblast lines, these results argue that the previously observed CI-MPR-independent killing mechanism was likely not representing grB-activity (Dressel et al., 2004a). As such, there is no basis to conclude that grB-mediated uptake and killing is CI-MPR-independent.

4.3. Discussion

The model that predicts the CI-MPR is a specific grB receptor has been a point of controversy over the last years. Following the initial evidence supporting this model (Motyka et al., 2000), data have accumulated to argue whether this model is irrelevant. Firstly, a CI-MPR-independent pathway was sufficient for grB-mediated killing (Trapani et al., 2003). Secondly, CTL-mediated killing was effectively induced irrespective of CI-MPR expression (Dressel et al., 2004a; Dressel et al., 2004b). Despite these dissenting claims, new data defend the model that, during granule-mediated killing, a grB-SG complex is released and, with the cooperation of pfn, induces apoptosis in a CI-MPR-dependent manner.

A renewed support for the CI-MPR-mediated grB uptake model was inspired by the observation that the pathway leading to grB-mediated killing was affected by specific co-factors during delivery to target cells. When grB was complexed to HS and delivered by pfn (figure 4-3c), or was complexed to SG and delivered by pfn or SLO (figures 4-1c and 4-4b, respectively), then grB-mediated killing was critically sensitive to M6P inhibition. In contrast, when grB was delivered in its free form by pfn or SLO (figures 4-1b and 4-3d, respectively), then grB-induced apoptosis was unaffected by M6P. However, the latter likely represents non-physiological conditions, given that granule-released grB has been detected as predominantly SG-bound (Galvin et al., 1999). Rather, these data suggest that under physiological conditions, grB uptake will likely be CI-MPR dependent, as depicted in figure 4-6a. Since CI-MPR-mediated endocytosis is dynamin-dependent (Willingham et al., 1981), this is consistent with previous indications that the grB-SG uptake pathway involves dynamin (chapter 3).

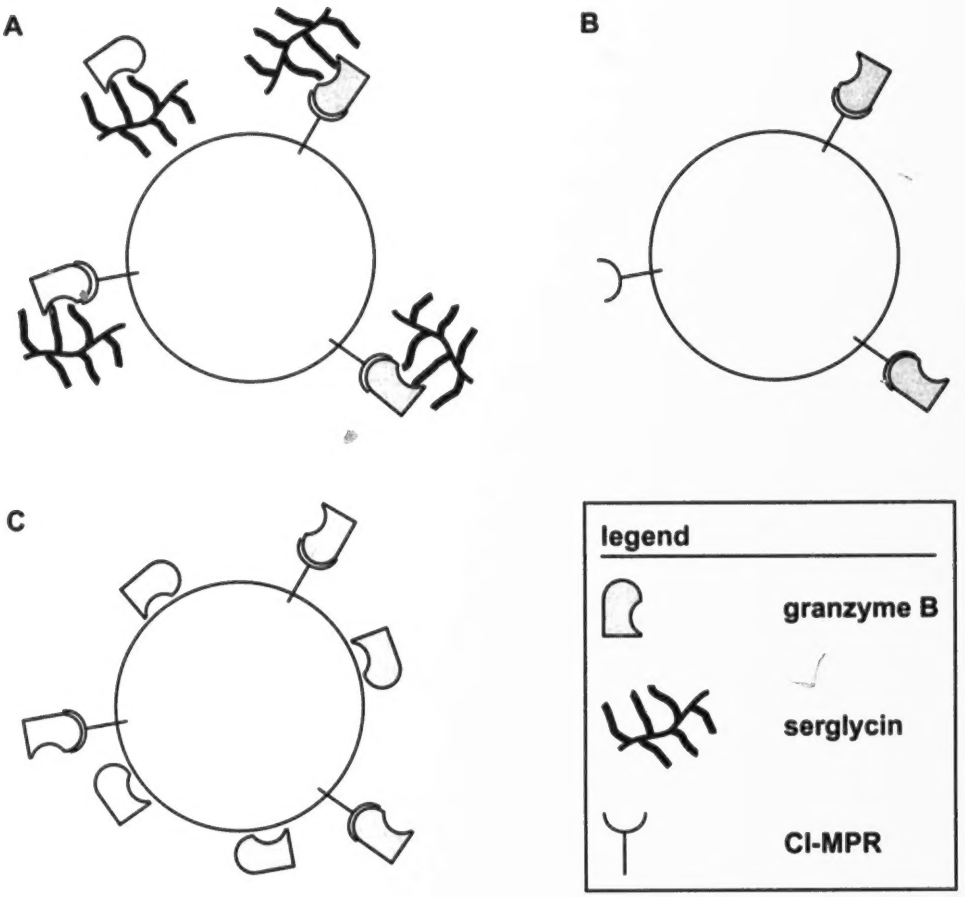

Figure 4-6. Granzyme B binding and uptake model.
A, Cell surface binding of grB-SG. *B*, Cell surface binding of free grB at low concentration. *C*, Cell surface binding of free grB at high concentration. GrB, via its M6P moiety, binds with high affinity to the CI-MPR. Low affinity binding is to negative charges on the cell surface (e.g. phospholipid headgroups, GAGs), via the intrinsic positive charge of grB. When bound to SG, the positive charge of grB is masked. The model predicts that when uptake of grB is mediated by the CI-MPR, then endocytosis also involves dynamin.

Regarding methodology, though the use of the GAGs heparin and heparan sulfate only loosely mimicked SG, the validity of their use is not unfounded. Firstly, free GAG chains can function in a manner similar to an intact proteoglycan. Complexing grB to CS-A chains (Galvin et al., 1999), like complexing to SG (Metkar et al., 2002), decreases the rate at which grB cleaves *in vitro* translated caspases 3 and 7. Secondly, different systems have suggested that grB will not only bind to CS-A chains, but also to heparin or heparan sulfate. Purification of grB has been assisted by its preferential binding to heparin-derived columns (Hanna et al., 1993), and grB has been found to bind to cell surfaces via heparan sulfate chains (Kurschus et al.). Finally, the results of the artificial grB-GAG and pfn killing model have been complemented by similar findings with grB-SG from degranulate material and SLO (figure 4-4b).

Beyond their validity as study tools, grB-heparin and grB-heparan sulfate complexes lend support to the predicted refining mechanism of SG. SG likely regulates the receptor-binding specificity of grB simply by forming a stable complex through charge-based interactions. To begin, SG binds to its cargo via a charge-dependent interaction, since dissociation is mediated by high concentrations of salt, but not by either non-ionic detergent or urea (Stevens et al., 1988; Kamada et al., 1989). This is consistent with the complementation of the negative charge of the CS-A side chains on SG (Dvorak et al., 1983; MacDermott et al., 1985; Parmley et al., 1985) and the positive charge of the grB molecule (Masson and Tschopp, 1987). Therefore this predicts that the positive charges on grB would be shielded against interactions with other negative charges on the surface of the cell (figure 4-6), such as GAGs and phospholipid headgroups. Notably, heparin and heparan sulfate likely bind to grB by a similar charge-based mechanism.

Since grB-heparin or grB-heparan sulfate complexes induced an effect comparable to that of grB-SG, then the use of these GAGs supports the hypothesis that SG refines the interactions of grB and receptors by shielding charges on grB.

To focus further on methodology, the most stringent test of the model involved degranulate material and SLO. A more ideal system would have involved degranulate material and pfn; however, in the absence of pfn, SLO is a more suitable choice than AdV. Killing with grB and AdV was exclusively MPR-dependent (figure 4-1a), while the particular pathway for killing with grB and pfn or SLO was affected by SG. Specifically, grB and pfn or SLO acted predominantly by an MPR-independent pathway in the absence of SG (figures 4-1b and 4-3d, respectively), and MPR-dependent in its presence (figures 4-1c and 4-4b). These patterns of grB binding and delivery have important implications for the delivery mechanisms of AdV, SLO and pfn. Free grB is likely able to access numerous binding sites on the cell surface, including the CI-MPR. AdV only has influence over grB delivered by the CI-MPR. In contrast pfn or SLO will easily interact with grB internalized from other sites, hence the observed CI-MPR-independent killing in their presence. But notably, when grB is preferentially directed to the CI-MPR by the chaperoning mechanism of SG, then pfn and SLO still may assist delivery of grB to the cytoplasm, indicating that these lytic agents have an overlapping, but greater range of activity when compared to AdV. So while it is unknown by what mechanisms pfn, SLO and AdV deliver grB, it is clear that SLO mimics pfn-mediated delivery more faithfully than AdV does.

Notably, the physiological relevance of the CI-MPR-mediated uptake model is based on the assumption that degranulated grB is predominantly SG-bound.

Considerable evidence suggests that, in degranulate, the major component is the grB-SG pool, and not the free grB pool. In degranulate materials, exclusively SG-bound grB was detected while free grB was below the limits of detection (Galvin et al., 1999). Further, estimated grB-SG affinities are similar at pH 5.4 and 7.4, which are representative of the granular and extracellular environments, respectively (Raja et al., 2002). But more remarkably, even after a drop in local grB concentration, as would occur following granule release, grB resists dissociation from SG and will even rebind (Raja et al., 2002). However, there may still be a pool of free grB. When grB-CS-A complexes were generated *in vitro*, a higher percentage of grB was found complexed to CS-A at pH 5.5 than at pH 7.4 (Galvin et al., 1999). Secondly, at pH 5.4, SG had a greater grB binding capacity than at pH 7.4 (Raja et al., 2002). Thus, if SG were saturated with grB upon granule formation, then upon granule release and neutralization, SG would be expected to release at least some of its grB due to its decreased binding capacity. Overall, the data suggest that if the degranulate material has a pool of free grB, it is likely small. Thus, it is likely that grB-SG is the physiologically relevant form of grB, and uptake will be CI-MPR-mediated under physiological conditions.

Though the present data appear to contradict some previous studies, in fact, the sum of the data supply interesting details to the model. The present study is the first to address the impact of SG on grB-mediated killing and its dependent on the CI-MPR, and so this provides an important distinction to the prior evidence. These new data also confirm prior detection of CI-MPR-independent uptake in the absence of SG (Trapani et al., 2003), which underscores the contribution of SG in focusing grB uptake via the CI-MPR. Notably, like previous studies of CI-MPR uptake, models for grB uptake by an

Hsp70 receptor (Gross et al., 2003) or by a receptor-independent pathway (Kurschus et al., 2005) are based on studies in the absence of SG. Arguably, these pathways may be physiologically irrelevant, since SG directed grB and pfn-mediated killing quantitatively through the CI-MPR pathway.

Strikingly, before the effect of SG on uptake was studied, previous evidence hinted at the nature of the CI-MPR uptake pathway. In particular, the induction of CI-MPR-independent uptake was dependent on high concentrations of grB (Trapani et al., 2003). The concentrations at which the effect was observed were in the range where significant grB binds non-specifically to the cell surface (Froelich et al., 1996). Thus these data suggest that the CI-MPR-dependent pathway is a high specificity, high affinity pathway, while the CI-MPR-independent pathway is of lower affinity (figure 4-6b,c). The present study complements these implications through the illustration that SG directs grB to the pathway of greater specificity.

Rationalization has contended with considerable evidence that contradicts CI-MPR-mediated grB-uptake. However, the *in vitro* data are challenged by the finding that CTL-mediated killing was equivalent in both wild type and CI-MPR knockout cell lines (Dressel et al., 2004a; Dressel et al., 2004b). To address this concern, the CTL-mediated killing of similar target cells, ConA-stimulated lymphoblasts, has been characterized to define the effector molecules. Remarkably, CTL-induced DNA fragmentation was not critically dependent on grB (figure 4-5a). This finding highlights the redundancy of CTL-mediated killing to ensure successful induction of apoptosis in modified target cells. But most relevantly, this argues that there is no evidence to support that grB uptake is CI-

MPR-independent in the previous CTL-mediated killing systems (Dressel et al., 2004a; Dressel et al., 2004b), so the previous claims are unfounded.

To consider a final concern about the CI-MPR-mediated uptake of grB, though it was predicted that SG might interfere with this pathway (Metkar et al., 2002), the opposite is true. Thus, SG does not mask the M6P binding determinant, nor does it create a steric barrier. Nonetheless, there is no indication of whether SG remains bound to grB at the receptor, or whether it might dissociate after ushering grB to the receptor.

The implications of the proposed model are several, as it pertains to future studies. It has already been inferred that the physiological relevance of further grB uptake studies may be judged based on the inclusion of SG or a suitable GAG analogue. The source of grB is also an important matter. It is becoming common practice to utilize granzymes from recombinant sources, due to more efficient recovery of materials, but this grB usually lacks the M6P moiety critical for binding to the CI-MPR. Thus in studies of granzyme uptake, recombinant materials should not be used as a measure of physiological events. This having been said, great insight could be gained through comparative analysis of grB from natural and recombinant sources. Further, alternative uptake routes may prove useful for therapeutic approaches.

The CI-MPR-mediated uptake model for grB may also apply to grA. This is first based on the structural similarity of grB to all granzymes (Masson and Tschopp, 1987). Secondly, grA possesses a M6P moiety (Burkhardt et al., 1989), which could facilitate binding to the CI-MPR. And finally, a stable grA-SG complex has been detected (Masson et al., 1990), implying SG might induce receptor-binding specificity similar to the scenario with grB.

In summary, this report presents data that provide new support for a much criticized grB uptake model, namely the CI-MPR-dependent pathway. A critical finding is that this is the preferred mechanism of uptake when grB is bound to SG. Though SG has typically been regarded as an inert molecule, SG has gained a new role through these studies, namely as a refiner of the grB uptake pathway.

Chapter 5

Granzymes A and B Maintain a Stable Complex with Serglycin after Secretion from Cytotoxic Granules

5.1. Introduction

During granule-mediated killing by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, the killer cells exocytose membrane-bounded granules, the contents of which induce apoptosis in target cells. The granule effectors that have been characterized most extensively include the serine proteases granzyme A (grA) and granzyme B (grB; Lieberman, 2003; Trapani and Sutton, 2003), both of which operate in cooperation with the pore-forming molecule perforin (pfn). Both granzymes induce apoptosis in the target cell by cleaving specific, but distinct cytoplasmic substrates. The current model describing granzyme access to these substrates predicts an endocytic step, followed by release from the endosome in a pfn-dependent manner (Froelich et al., 1996).

The cytotoxic granules not only house and secrete the cytotoxic molecules, but also a proteoglycan, serglycin (SG; Kolset et al., 2004). The peptide core of serglycin is distinguished by repeating serine-glycine units (Bourdon et al., 1985; Bourdon et al., 1986), which are the attachment sites for *O*-linked glycosaminoglycans, specifically chondroitin sulfate A (CS-A) side chains in CTL and NK cells (Dvorak et al., 1983; MacDermott et al., 1985; Parmley et al., 1985). An intracellular function is known for SG, since it is required for the formation of mature granules in mast cells (Forsberg et al., 1999; Humphries et al., 1999; Åbrink et al., 2004). Consistent with a similar packing role in CTL and NK cells, SG has been found to form stable complexes with grA (Stevens et al., 1988; Kamada et al., 1989), grB (Metkar et al., 2002) and pfn (Masson et al., 1990) inside granules. These interactions are predominantly charge-dependent, since

complexes are destabilized by high concentrations of salt, but not by detergent or urea (Stevens et al., 1988; Kamada et al., 1989).

In contrast to a clear intracellular role, less is known about the extracellular function of SG. We now suggest that an extracellular function for SG may be to refine the uptake pathway of grB during granule mediated-killing (chapters 3 and 4). According to the model, during pfn-assisted killing, grB-SG is internalized by the cation-independent mannose 6-phosphate receptor (CI-MPR) via dynamin-dependent endocytosis, while free grB lacks specificity for the CI-MPR. As such, characterization of grB-SG complexes would build on this model, particularly with respect to its physiological relevance.

It is known that SG can remain complexed to some of its cargo following release from granules. For instance, both grA-SG (Masson et al., 1990) and grB-SG (Galvin et al., 1999) complexes have been detected in degranulate of killer cells. It is also known that some cargo dissociate from SG following degranulation (Masson et al., 1990; Ghildyal et al., 1996). Thus a key point of interest is the degree to which granzymes remain bound to SG after degranulation.

Regarding the nature of SG complexes , it was observed that a SG-mediated physical link was detected between pfn and grB (Metkar et al., 2002; Raja et al., 2002). This provides evidence that a single SG molecule binds a mix of cargo. Applying this finding to uptake models, this implies that when one of the complexed molecules binds to a specific receptor, a whole array of cytotoxic molecules are internalized due to the physical association with the SG complex. Such a hypothesis makes two untested assumptions. Firstly, it presupposes that after the cytotoxic molecule binds to the

receptor, SG will remain bound. The second presumption is that all types of cytotoxic molecules are physically linked by binding to SG.

To contribute to the developing model describing SG function, granzyme-SG complexes have been characterized. Experiments have been conducted to test conditions that affect the relative stability of grA- and grB-SG complexes. Further, it has been assessed whether CI-MPR might internalize granzyme-SG complexes. Overall, evidence suggests that grB-SG is a major factor in degranulate material, and that an intact complex can associate with the CI-MPR.

5.2. Results

5.2.1. GrA- and grB-SG complexes can be analyzed over a 100-kDa cut-off filter

With the aim to better characterize the grA- and grB-SG complexes, human CTL (hCTL) degranulate material was chosen as a convenient source of these complexes. In order to stimulate degranulation, hCTL were incubated over immobilized anti-CD3 for 4 h at 37°C. The degranulate material was then treated under various conditions that might affect the stability of the complex. The pools of free or SG-complexed granzymes were physically separated, based on previous methods (Galvin et al., 1999). Degranulate material was fractionated over a 100-kDa cut-off centrifugal filter, so that free granzyme, a 50 kDa grA homodimer (Poe et al., 1988) or 32 kDa grB monomer (Poe et al., 1991), would partition in the low-molecular weight filtrate, and the SG-complexed granzymes in the high-molecular weight retentate. Since equal volumes of the fractions were analyzed, the concentrated retentate and the filtrate materials were compared to the starting material

to assess the states of the granzymes. Specifically, in the retentate, SG-complexed granzymes were implied when granzyme concentrations were greater than the starting material. In the filtrate, the free granzyme pool was at concentrations less than or equal to the starting material, such that a direct comparison gave a proportional measure of the pool size.

To assess this experimental system, prior to fractionation, degranulate was either untreated, or treated to destabilize complexes. Granzyme-SG complexes were destabilized for the control assay by incubating degranulate in a pH 4.0 buffer with 390 mM sodium chloride on ice for 1 h. This treatment was chosen since these conditions have been applied to solubilize granzymes away from SG in isolated, delipidated granules (Hanna et al., 1993). Partitioning of grA or grB was monitored by immunoblot analysis (figure 5-1a,b).

When grA was screened (figure 5-1a), in the untreated sample grA concentrated primarily in the high-molecular weight retentate fraction, and was barely detectable in the low-molecular weight filtrate fraction. In contrast, the acidic, high salt treatment led to nearly equivalent partitioning of grA between the retentate and filtrate. A similar profile was observed when samples were probed for grB (figure 5-1b). These data implied that the filtration method could separate SG-complexed and free granzyme pools.

In order to further test the hypothesis that the filter was specifically retaining grB-SG complexes, degranulate material was analyzed by gel filtration chromatography as a complementary approach. The sample was loaded and eluted in 1% CHAPS buffer, then grB was tracked by enzymatic activity. Two pools of distinct molecular weight were detected (figure 5-1c). The first pool was less than 43 kDa, which is consistent with the

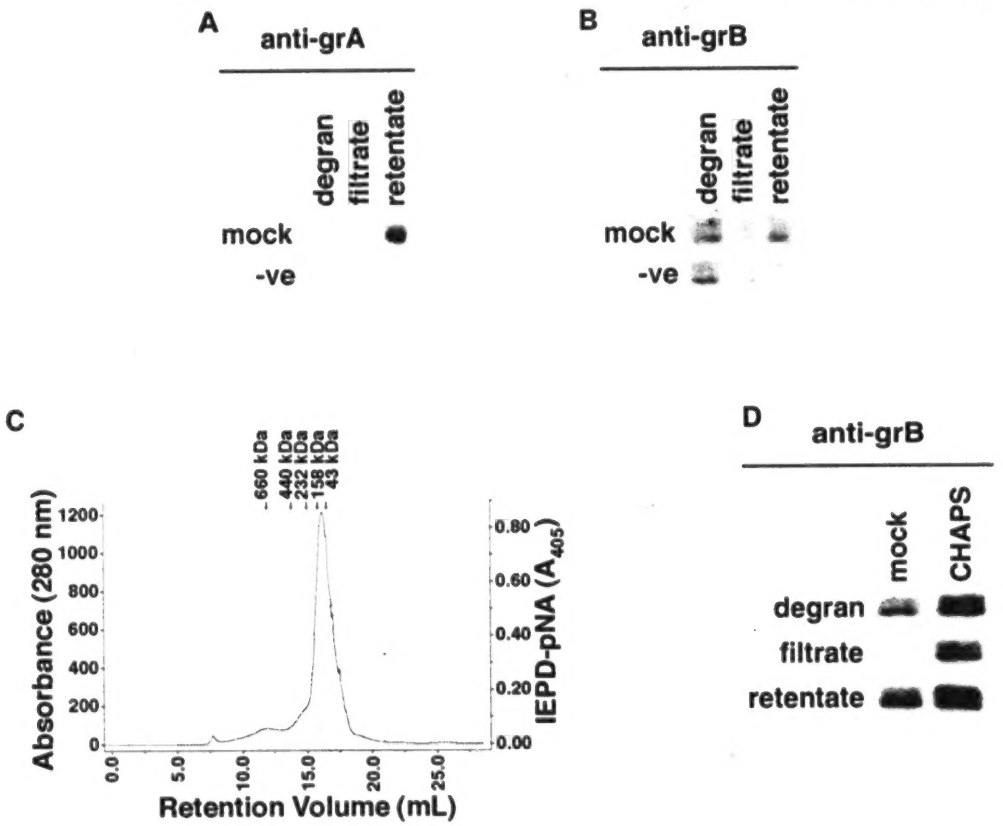

Figure 5-1. Granzyme-SG complexes can be separated from free granzyme over a 100-kDa cut-off filter.

Degranulate was obtained by incubating CTL over immobilized anti-CD3 for 4 h at 37°C. *A,B*, Degranulate material was fractionated over a 100-kDa cut-off filter immediately (mock), or after treatment with 40 mM sodium acetate, pH 4.5, and 390 mM NaCl for 1 h on ice (-ve) to dissociate granzyme-SG complexes. Equal volumes of samples were analyzed by immunoblot to detect grA (A) or grB (B). *C*, Anti-CD3-induced CTL degranulate from 2×10^8 cells, diluted in CHAPS buffer (20 mM Tris, pH 7.4, 137 mM sodium chloride, 1% CHAPS), was resolved by gel filtration on a Superose 6 10/30 column with a flow rate of 0.5 mL/min. GrB activity was detected in the 0.5 mL fractions by IEPD-pNA colorimetric assay. Solid line, A_{280}; broken line, IEPD-pNA cleavage activity (A_{405}). *D*, CTL degranulate was treated with 50 mM Tris, pH 7.5, in the absence (mock) or presence (CHAPS) of 1% CHAPS for 1 h at 37°C. Samples were fractionated over a 100-kDa cut-off filter, and equal volumes of samples were analyzed by immunoblot to detect grB. Data are representative of three (A,B), one (C) or two (D) independent experiments.

32 kDa monomeric form of grB. The second grB-containing pool was ~650 kDa, which is consistent with a large SG-containing complex, and, importantly, indicates that grB can be found in a large molecular weight complex following degranulation. Notably, the pool of free grB was significantly greater than the SG-bound pool. Similarly, in a control filtration assay, degranulate treated with 1% CHAPS had a large pool of free grB (figure 5-1d). This suggests that CHAPS was destabilizing the grB-SG complex, thus these data could not be used as a measure of relative pool sizes in the degranulate material. Notably, the similar results between the two methods defend the use of the filter fractionation method for further analysis of samples. This is further supported by previous findings that free grB passes freely through a 100-kDa cut-off filter (chapter 4).

One interesting observation from filter fractionation in the presence and absence of CHAPS is that there is greater recovery of grB in the CHAPS-treated sample (figure 5-1d). This would imply that, normally during degranulate treatment, some grB is lost in the sample tube. The specific nature of the lost material, whether it represents overall non-specific loss or preferential loss of free grB, is unknown.

5.2.2. CTL secrete grA- and grB-SG complexes in response to allogeneic targets

An additional concern for this experimental method was whether anti-CD3-induced degranulation gave a reasonable representation of target-induced degranulation. The specific interest was whether targets induced release of granzyme-SG complexes. This was examined by incubation of hCTL with the target cell line Jurkat, and degranulates were filter fractionated, then analyzed for the enzymatic activities of grA or

grB (Table 5-1). To normalize the granzyme detected in the retentate material, the estimated concentration was divided by the concentration factor of the retentate sample. Release of grA from hCTL required stimulation by targets, though some grB was released spontaneously. In the target-induced degranulate, the predominant form of either granzyme was high-molecular weight. This indicated that SG-complexed granzymes are released from granules in response to both target- and anti-CD3-mediated stimulation of killer cells.

Notably, though the reported concentrations of grB suggested significant loss during processing, likely the relatively low concentration of grB in the retentate is an artifact of the estimation method. Specifically, the high concentration of grB in the retentate laid in the non-linear range of the standard curve, thus decreasing the accuracy of the interpolated grB concentration. In support of this, in a single experiment the retentate fraction was normalized by diluting with buffer, and by this method the estimated grB concentrations indicated reasonable recovery of grB (data not shown).

5.2.3. Characterization of granzyme-SG complexes with respect to time, pH, and freeze-thaw cycles

The relative stability of granzyme-SG complexes has become an important question because of evidence that SG affects the targeting of grB to cell surface receptors (chapters 3 and 4). One concern is the relative pool sizes of free versus SG-bound grB following degranulation. Degranulation leads to changes in pH, for instance, and this change might have destabilizing effects on the granzyme-SG complexes. Other concerns surround handling conditions that will affect the quality of the degranulate material, such as stability over time, and freeze-thaw cycles.

Table 5-1. GrA and grB-SG complexes are present in target-induced CTL degranulate material.

CTL were incubated with Jurkat cells (1:1 E/T) at 37°C for 4 h to induce degranulation. Co-culture supernatant, containing the degranulate, was fractionated over a 100 kDa cut-off filter. Granzyme activity in each sample was quantified by BLT esterase (grA) or IEPD-pNA (grB) colorimetric assays, then granzyme concentrations (ng/mL) were estimated based on a standard curve analyzed in parallel; retentate samples were normalized by dividing the concentration of granzyme activity found in the retentate by the concentration factor of the fraction. The mean ± SD of three independent experiments is shown. For both reaction set-up and fractionation, $P < 0.01$ for grA samples, and $P < 0.0001$ for grB samples according to matched two-way ANOVA analysis.

		Cell mixture		
granzyme	fraction	CTL	Jurkat	CTL + Jurkat
grA	degranulate	bld[a]	bld	64 (±30)
	filtrate	bld	bld	bld
	retentate	bld	bld	61 (±37)
grB	degranulate	124 (±60)	bld	199 (±69)
	filtrate	bld	bld	bld
	retentate	bld	bld	67 (±22)

[a] bld, below level of detection

In order to analyze the stability of granzyme-SG complexes in the degranulate material, samples were incubated for up to 24 h at 37°C, in a 5% CO_2 environment to maintain neutral pH of the medium. At the indicated time-points, samples were fractionated over the 100-kDa cut-off filter, then the grA or grB in the fractionated materials were detected by immunoblot (figure 5-2a,b). The granzyme molecules themselves were stable, since both grA and grB were consistently detected in the starting materials (degran), even up to 24 h. Fractionation revealed that the granzymes predominantly partitioned to the retentate, suggesting secreted granzymes are predominantly SG-bound. Since the fractionation profile was maintained over the 24 hours, this suggested that the steady state levels of free and SG-bound granzyme were stable under these incubation conditions, and that time alone will not affect complex stability.

The next point of interest was the relative effect of pH on the granzyme-SG complexes, since this may not only influence granzyme-SG complex stability following degranulation, but is also a controllable variable in assays. The effect of pH over the range of pH 4.5 to 9.0 was investigated by adding various buffers to degranulate samples, then the samples were incubated at 37°C for 1 h before fractionation over the filter (figure 5-2c,d). Immunoblots of the starting samples (degran) showed that neither the grA nor grB molecules proper are degraded at any pH tested. Following fractionation, in general, either granzyme partitioned predominantly to the retentate fraction, and much less to the filtrate, suggesting the granzyme-SG complexes were relatively stable. However the relative intensities of the retentate and filtrate fractions did reveal that pH affected the relative size of the granzyme-SG pool in nuances.

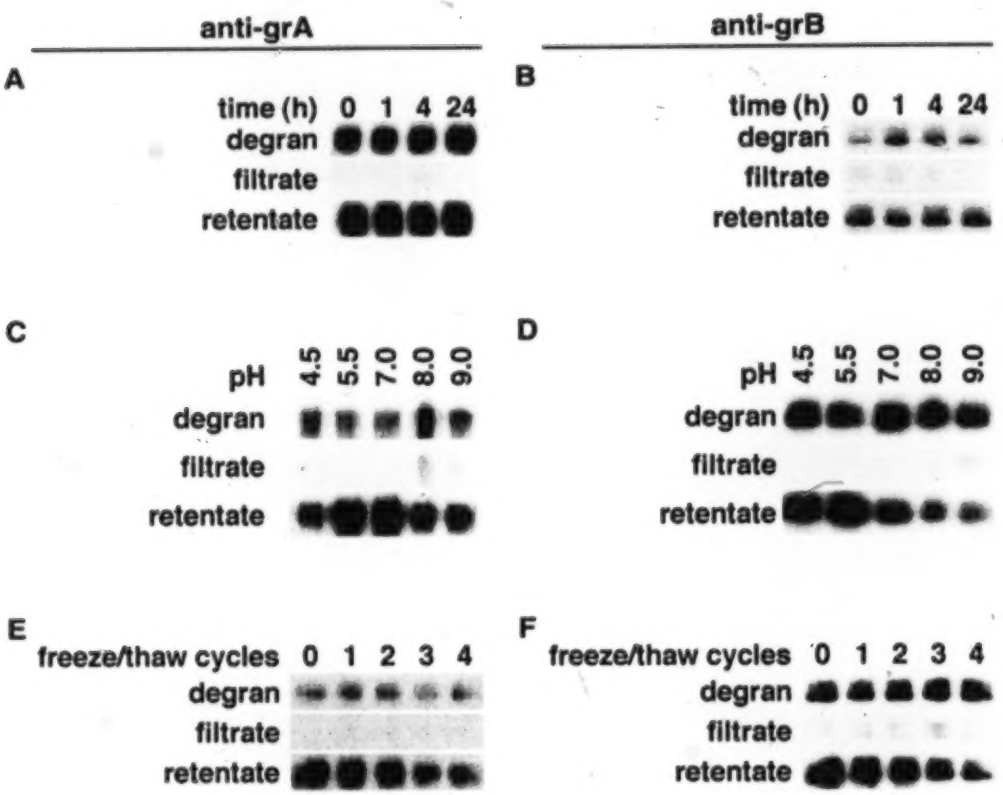

Figure 5-2. Characterization of granzyme-SG stability in CTL degranulate. Degranulate was obtained by incubating CTL over immobilized anti-CD3 for 4 h at 37°C. After the indicated treatments, samples were fractionated over a 100-kDa cut-off filter. Equal volumes of samples were analyzed by immunoblot to detect grA (A,C,E) or grB (B,D,F). *A,B*, time-course. Degranulate was incubated at 37°C in a 5% CO_2 atmosphere for the indicated hours, and fractionated at time-points. *C,D*, pH effect. To degranulate samples, the following buffers were added to a final concentration of 50 mM: sodium acetate, pH 4.5; MES, pH 5.5; HEPES, pH 7.0; Tris, pH 8.0; boric acid, pH 9.0. Samples were incubated 1 h at 37°C. *E,F*, freeze-thaw effect. Samples were subjected to the indicated number of freeze-thaw cycles. One cycle allowed freezing for at least 30 min at -80°C, followed by rapid thaw in a 37°C water-bath. Data are representative of at least three independent experiments.

For grA (figure 5-2c), the most intense retentate bands, and conversely least intense filtrate bands were at pH 5.5 and 7.0. At pH 4.5, the grA band in the retentate was moderately decreased, and at pH 8.0 and 9.0, the intensity was further diminished, though still by modest amounts; in each case the intensity of the grA bands in the filtrates was complementary. These data suggested that grA binds to SG most effectively at pH 5.5 and 7.0, but at the more extreme levels of pH, some grA dissociates. The comparable binding at pH 5.5 and 7.0 is consistent with previous studies (Masson et al., 1990).

For grB (figure 5-2d), at pH 4.5 and 5.5, the retentate band was most intense, then as the pH increased from 7.0 through 9.0, grB decreased in the retentate. Detection of grB in the filtrate was complementary. These results suggested that SG binds grB more efficiently at pH 5.5 or less, but that at pH 7.0 grB begins to dissociate. This is consistent with SG possessing different capacities for binding grB at pH 5.4 and 7.4 (Raja et al., 2002).

The final issue explored was the effect of freeze/thaw cycles on granzyme-SG complex stability. While this is unimportant for the physiological model, it was relevant experimentally since extended storage of degranulate stocks required freezing in order to maintain pH. To test this parameter, samples of degranulate were subjected to numerous freeze-thaw cycles, from −80°C to 37°C, before fractionation over the filter. Immunoblots for either grA (figure 5-2c) or grB (figure 5-2f) showed that the stability of the granzyme-SG complexes decreased as the number of freeze-thaw cycles increased. Importantly, by the third freeze-thaw cycle, the granzymes detected in the retentate samples were significantly diminished, suggesting significant reduction in the granzyme-SG pools. These findings are consistent with the use of freeze-thaw in procedures used to

isolate granzyme from isolated granules (Shi et al., 1992b). But more importantly, this highlights a limitation of handling these materials.

5.2.4. Perforin-SG complexes are destabilized by extracellular conditions

Pfn, like grA and grB, forms a stable complex with SG during storage in acidic granules (Masson et al., 1990; Metkar et al., 2002), thus, it was possible that pfn would bind to SG under the neutral conditions of the degranulate material. CTL degranulate was incubated for up to 24 h at 37°C in a 5% CO_2 chamber to maintain the neutral pH of the media. At the indicated time points, samples were filter fractionated, then pfn was detected by immunoblot (figure 5-3). Importantly, a weak band of consistent intensity was detected in the treated degranulate samples (degran) up to 24 h, indicating that the pfn molecule was not degraded over the course of this assay. At all time points, pfn levels in the filtrate were below detection. Distinctly, at 0 h pfn concentrated into the retentate fraction, but was less evident in this fraction as time progressed. This suggested that pfn had been detected in a high-molecular weight complex that dissociated over time. This complex might reflect pfn binding to SG, though the data would not distinguish from pfn that had polymerized in solution due to Ca^{2+} exposure. Assuming that the detected labile complex is SG-pfn, this would be consistent with previous detection of the pfn-SG complex at neutral pH (Metkar et al., 2002), as well as with reports that the complex is unstable under these conditions (Masson et al., 1990; Metkar et al., 2002).

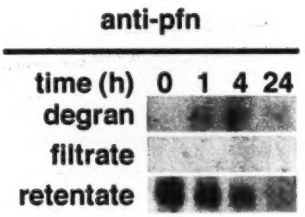

Figure 5-3. A high-molecular weight pfn complex is sensitive to time.
Degranulate was obtained by incubating CTL over immobilized anti-CD3 for 4 h at 37°C. Degranulate was incubated at 37°C in a 5% CO_2 atmosphere for the indicated hours, and at time-points, samples were fractionated over a 100 kDa cut-off filter. Equal volumes of samples were analyzed by immunoblot to detect pfn. Data are representative of three separate experiments.

5.2.5. GrA and grB bind to CI-MPR via a M6P moiety, and can bind while complexed to SG

Based on uptake models for grB, as well as the similarities of grA and grB (Masson and Tschopp, 1987), an attractive model is that grA, delivered by killer cells, might utilize the CI-MPR as an internalization receptor on the surface of the target cell. Also, since grA possesses a M6P moiety (Burkhardt et al., 1989) which is involved in trafficking grA to the cytotoxic granule (Griffiths and Isaaz, 1993), this indicates that grA would have the potential to bind to the CI-MPR on the surface of the target cell.

To first probe for an interaction between grA and CI-MPR, a biotinylated soluble form of the CI-MPR (bCI-MPR) was used to probe for grA by Far Western. As a further test of specificity, prior to SDS-PAGE analysis, a sample of purified grA was treated with Endo H or with calf intestine phosphatase to damage the M6P moiety (figure 5-4a). Note that as a control of phosphatase activity, treatment was performed in the presence and absence of inorganic phosphate to inhibit phosphatase activity (Motyka et al., 2000). As a further control, based on evidence of interactions between grB and the CI-MPR (Motyka et al., 2000), grB samples were analyzed in parallel (figure 5-4b). Immunoblots of EndoH-treated grA or grB showed that deglycosylation essentially reached completion, since the treated granzymes migrated much faster than those that were mock-treated. Similarly, phosphatase treatment affected migration of both grA and grB by causing slightly faster mobility, and notably this effect was prevented in the presence of inorganic phosphate. When parallel blots were probed with bCI-MPR, interactions were detected with both grA (figure 5-4a) and grB (figure 5-4b) in mock-treated samples, or where phosphatase activity was blocked by inorganic phosphate. In contrast, where the

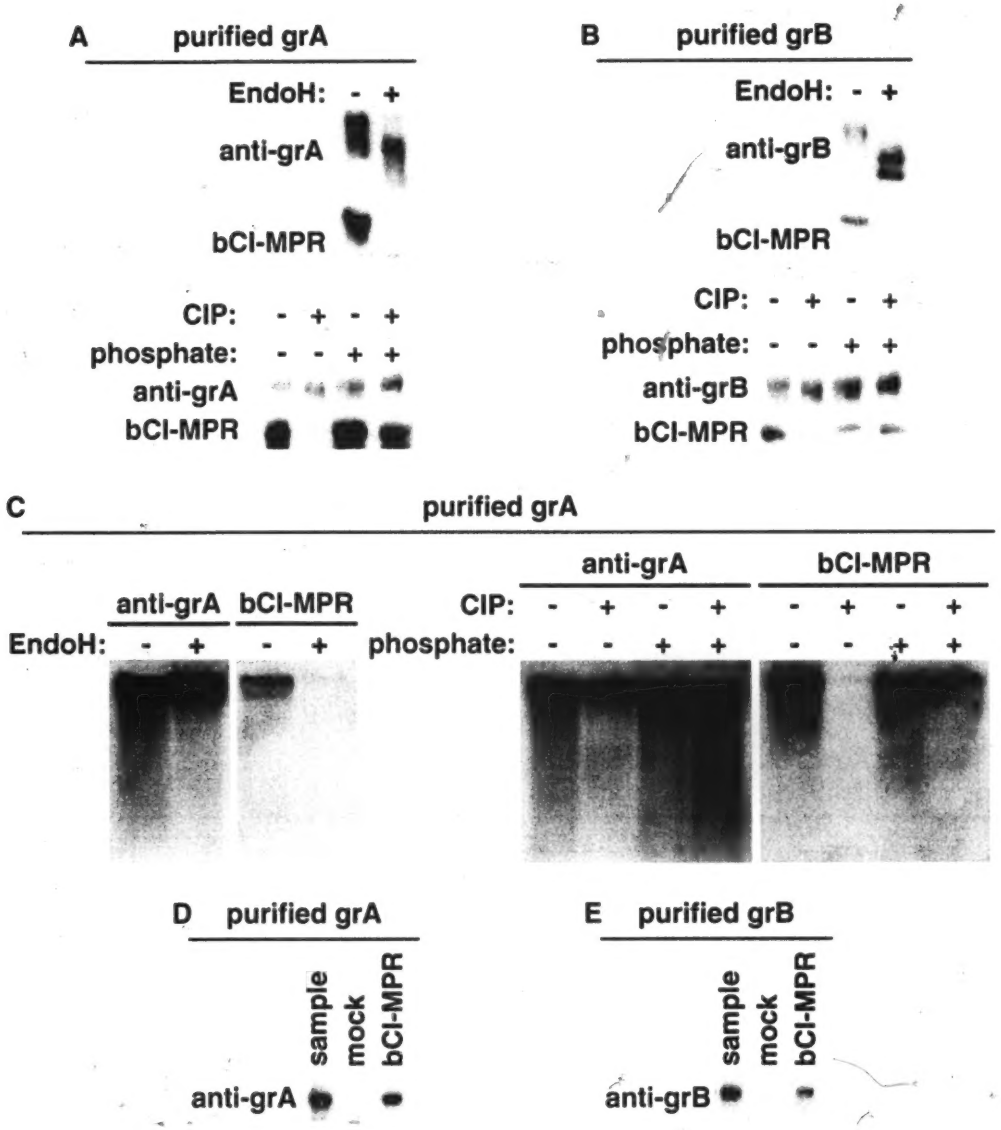

Figure 5-4. grA or grB specifically binds to the CI-MPR via its M6P moiety, and can bind while complexed to SG.

A,B,C, purified grA (A,C) or grB (B) was pre-treated with EndoH, or calf intestine phosphatase (CIP) in the presence or absence of 85 mM phosphate buffer to inhibit the phosphatase. Samples were resolved by SDS-PAGE (A,B) or native agarose (C), then analyzed by immunoblot with an anti-grA (A,C) or anti-grB (B) antibody, or by Far Western with a biotinylated, soluble form of the CI-MPR (bCI-MPR). *D,E*, a solution of purified grA (D) or grB (E) was incubated with Neutravidin-agarose beads alone (mock) or with beads allowed to pre-bind bCI-MPR. Pelleted material was probed by immunoblot to detect grA (D) or grB (E).(continued)

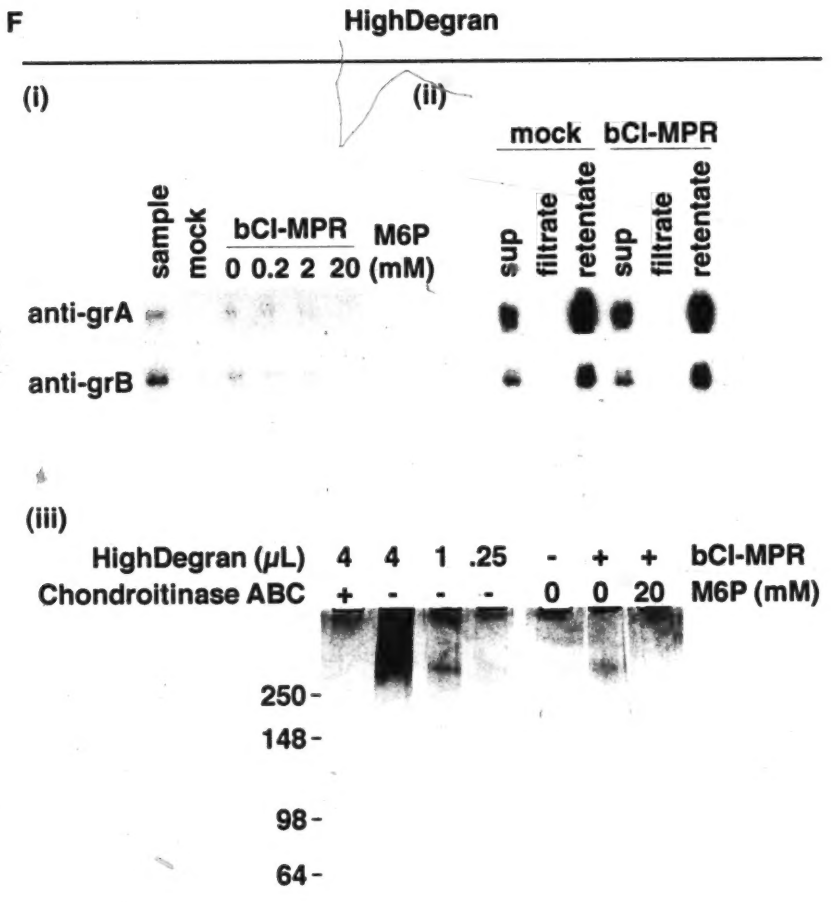

Figure 5-4, continued.

F, Degranulate was obtained by incubating CTL over immobilized anti-CD3 for 4 h at 37°C. To obtain a fraction of predominantly SG-complexed granzymes, the degranulate was fractionated over a 100-kDa cut-off filter, and the high molecular weight retentate (HighDegran) was recovered. HighDegran was incubated with bCI-MPR pre-bound to Neutravidin-agarose beads. In some samples, mannose 6-phosphate (M6P; 0.2 to 20 mM) was present during incubation. (i) Pelleted material was probed by immunoblot to detect grA or grB. (ii) Supernatants of samples were recovered and fractionated over a 100-kDa cut-off filter. Equal volumes of samples were analyzed by immunoblot to detect grA or grB. (iii) Pelleted material was resolved on a 4-15% gradient SDS-PAGE, and sensitivity-enhanced silver staining was performed to detect proteoglycans. As controls to identify SG, HighDegran was treated with or without chondroitinase ABC, and was analyzed in titrated amounts. Data are representative of three independent experiments.

M6P had been damaged, whether by EndoH or phosphatase treatment, no receptor-granzyme interaction was observed. These data suggested that grA, like grB, can interact with the CI-MPR and that this interaction is specifically dependent on the M6P moiety on the granzyme molecules.

In addition, the grA samples were resolved on a non-denaturing agarose gel to maintain the native structure of the molecule, and once again probed by Far Western blot with the bCI-MPR (figure 5-4c). Since grA is a basic protein and thus positively charged, grA migrates toward the cathode in non-denaturing gels. As was observed for the SDS-PAGE blots, bCI-MPR interacted with grA where the M6P moiety was left intact, but did not bind where it was destroyed by either EndoH or phosphatase treatment. This suggested that in native form, grA can bind specifically to the CI-MPR in a M6P-dependent manner.

In the inverse approach of this experiment, bCI-MPR was first bound to Neutravidin-Agarose beads, then incubated with solutions of purified grA, or, as a control, grB. An immunoblot for the granzymes in the pelleted materials revealed that grA (figure 5-4d), like grB (figure 5-4e), bound stably to the CI-MPR. Importantly, this revealed that in solution, grA could bind to the CI-MPR. This is consistent with mannose 6-phosphate receptors binding to grA for the purposes of trafficking to the granule (Griffiths and Isaaz, 1993), however, it was important to test the interaction under these assay conditions.

Thus far, the data suggest that grA and grB can form a stable interaction with the CI-MPR. A remaining concern was whether these granzymes, when complexed to SG, would still be able to bind the receptor. Though it has been suggested that grB-SG is

preferentially targeted to the CI-MPR (chapter 4), there has been no test of whether SG remains bound after grB binds to the receptor. In order to address this, hCTL degranulate was collected, then fractionated on a 100-kDa cut-off filter to obtain a concentrated source of high molecular weight granzyme-SG complexes (HighDegran). Then bCI-MPR pre-bound to Neutravidin-Agarose beads was incubated with the HighDegran, and the pellet material was probed by immunoblot for grA or grB. As seen with the purified granzymes, grA and grB were precipitated in a bCI-MPR-dependent manner (figure 5-4 f(i)). Further, these interactions were found to be M6P-dependent, since increasing concentrations of M6P sugar in the sample prevented the binding of grA and grB to the bCI-MPR beads. Notably, these data showed that the degranulated grA and grB still retained their M6P modifications, and not that the M6P had been removed within the cytotoxic granules. But primarily, these observations implied that granzyme bound to SG could interact with the CI-MPR, consistent with previous findings (chapter 4). However, these data alone did not test whether intact granzyme-SG could interact with the CI-MPR.

To examine whether SG remained complexed with granzyme that had bound the CI-MPR, granzyme-SG complexes were first screened for their relative stability throughout the incubation period. Following co-incubation of HighDegran and bCI-MPR beads, supernatants from the samples were retrieved, then fractionated over the 100-kDa filter. Immunoblots probing for either grA or grB showed that they partitioned in a very concentrated manner to the retentate, and in the filtrate granzymes were below levels of detection (figure 5-4f(ii)). This suggested that overall the granzyme-SG complexes were stable throughout the incubation period. Even so, it was likely that the association between granzymes and SG was dynamic, and as such, granzymes in their unbound state

might preferentially be sequestered by the bCI-MPR. Therefore bCI-MPR-associated granzymes were assessed for SG interactions by resolving pelleted materials on a gradient SDS-PAGE, and subjecting it to sensitivity-enhanced silver staining to detect proteoglycan (figure 5-4f(iii)). In the precipitated materials, a band greater than 250 kDa was observed that was specifically dependent on bCI-MPR. While the molecular weight of the detected entity was consistent with that of SG (Raja et al., 2002), as a specificity control, HighDegran was analyzed in parallel after mock and chondroitinase ABC treatment. Indeed, disappearance of the band after digestive treatment indicated that it was a proteoglycan modified with chondroitin sulfate side chains, and was most likely SG. Thus, SG was retrieved in a CI-MPR-dependent manner. Further, this interaction was competed by 20 mM M6P, suggesting that the interaction was dependent on binding via a M6P moiety. The data do not rule out the possibility that SG is interacting directly with the CI-MPR via the receptor's M6P binding site, though this seems unlikely due to the absence of M6P tags on SG. Rather, the most likely interpretation of these data is that SG is co-precipitated with granzymes, implying that the trimer SG-granzyme-CI-MPR may form. Notably, any other CI-MPR-binding factor present in the HighDegran also could behave as the molecular bridge between SG and the receptor.

5.2.6. GrA and GrB bind to distinct pools of SG within the degranulate mixture

Since there is evidence to suggest that either grA or grB might bind to the CI-MPR while still bound to SG, this raises the question of whether an array of granzymes would be physically linked to a single receptor, with SG serving as a molecular bridge. This issue has also been raised by the co-precipitation of grB and pfn from isolated granules (Metkar et al., 2002), as well as the co-detection of pfn in grB-SG complexes

from isolated granules (Raja et al., 2002). Notably, there has been no exploration of whether grA and grB are linked by SG-association.

In order to explore this possibility, biotinylated anti-grA or anti-grB antibodies were pre-bound to Neutravidin-Agarose beads, then incubated with HighDegran. Materials isolated by centirfugation were screened by immunoblotting for grA or grB. First, it was established through precipitation of purified grA or grB materials (figure 5-5a) that the antibodies interact exclusively with their specified granzyme antigen, consistent with previous evidence (Spaeny-Dekking et al., 1998). Next, when HighDegran was immunoprecipitated, similarly each antibody only retrieved its specific antigen. This implied that grA and grB were not physically linked, though it was not yet clear whether this was due to dissociation from SG, or whether these molecules were associated with distinct pools of SG. To assess these possibilities, first, the immunoprecipitates were screened for the presence of SG. Indeed, silver stain analysis indicated that SG was associated with the immunoprecipitated granzymes (figure 5-5b). Second, analysis of the sample supernatants showed within the limits of detection that the granzymes remained preferentially bound to SG throughout the incubation, irrespective of incubation conditions (figure 5-5c). Since granzymes were found to co-precipitate with SG, yet grA did not co-precipitate with grB or vice versa, this suggested that grA and grB are segregated to distinct pools of SG, rather than being packed onto shared molecules of SG.

One interesting observation was that immunoprecipitation depleted grA in a near-quantitative manner from the HighDegran sample supernatant (figure 5-5c). This is relevant because ultrastructural studies of granules have detected a second membrane

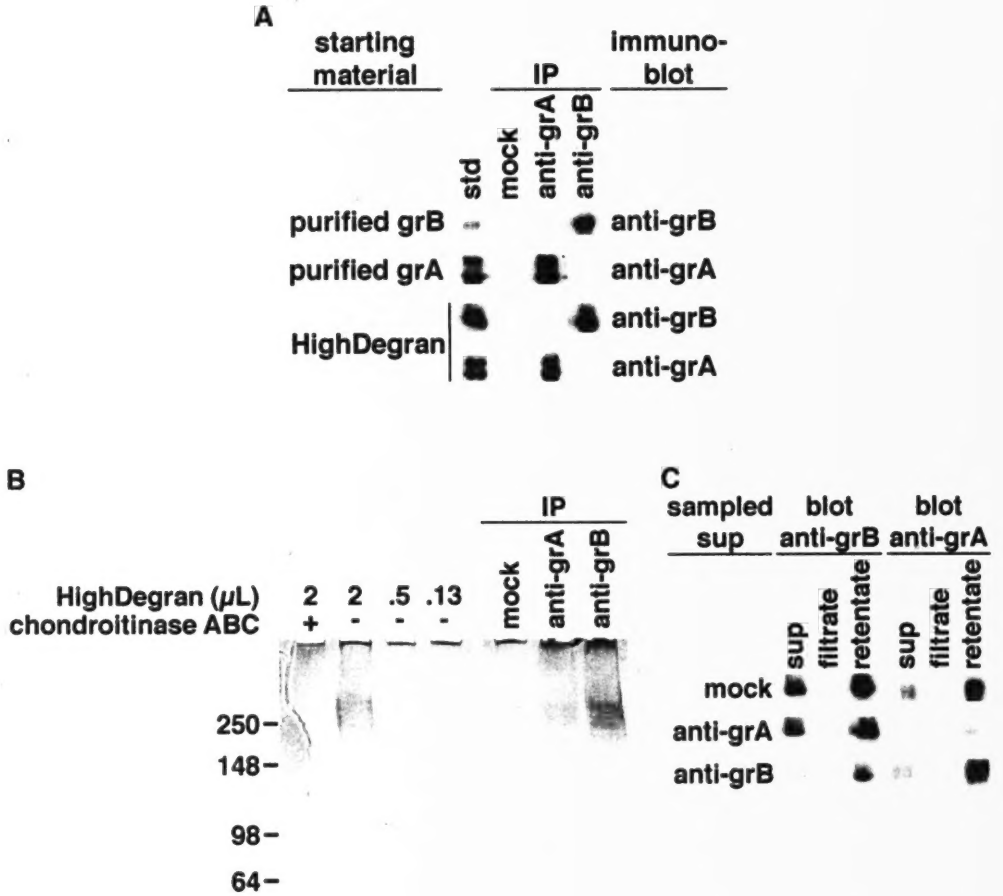

Figure 5-5. Immunoprecipitation of grA-SG complexes does not co-precipitate grB, and vice versa.

Degranulate was obtained by incubating of CTL over immobilized anti-CD3 for 4 h at 37°C. To obtain a fraction of predominantly SG-complexed granzymes, the degranulate was fractionated over a 100-kDa cut-off filter, and the high molecular weight retentate (HighDegran) was recovered. Purified grA, purified grB, or HighDegran were incubated with Neutravidin-agarose beads alone (mock), that had pre-bound biotinylated anti-grA, or that had pre-bound biotinylated anti-grB. *A*, Pelleted material was probed by immunoblot to detect grA or grB. *B*, Pelleted material was resolved on a 4-15% gradient SDS-PAGE, and sensitivity-enhanced silver staining was performed to detect proteoglycans. As controls to identify SG, HighDegran was treated with or without chondroitinase ABC, and was analyzed in titrated amounts. *C*, Supernatants of samples were recovered and fractionated over a 100-kDa cut-off filter. Equal volumes of samples were analyzed by immunoblot to detect grA or grB. Data are representative of three independent experiments.

surrounding the granule core, which houses SG, granzymes and pfn (Peters et al., 1989; Burkhardt et al., 1990). Thus one proposal has been that after exocytosis of granules, the cytotoxic molecules are still contained by a membrane. However, since the secreted grA was accessible for immunoprecipitation despite an absence of detergent, this would argue that the secreted cytotoxic molecules are not enclosed in a membrane.

5.3. Discussion

An extracellular role for SG has long since been predicted by its secretion during granule-mediated killing (Levitt and Ho, 1983; Schmidt et al., 1985). Despite this long-standing observation, a model describing function has been lacking. Recent evidence has suggested that SG may refine the pathway by which grB is endocytosed into the target cell (chapters 3 and 4). Physiological relevance of this model is supported by detection of grB-SG (Galvin et al., 1999), as well as grA-SG (Masson et al., 1990) complexes in degranulate material. The present study provides further support for the model that, following granule release, grB is predominantly SG-bound. Expolaration has also revealed that, in particular, the intact granzyme-SG complex can bind to the CI-MPR, thus, under physiological conditions, the CI-MPR likely will mediate the internalization of SG-complexed granzyme. Notably, internalization of the intact SG complex will not mediate co-delivery of grA and grB.

Since CI-MPR- and dynamin-specific grB endocytosis require that grB is complexed to SG, the overall stability of grB-SG complexes is a relevant matter. In particular, complex stability upon granule release is a point of interest, because this event

causes a change in pH from roughly 5.5 to 7.4, as well as a dilution of granule factors. Either of these factors might theoretically induce dissociation of granzyme-SG complexes. Indeed retention and dissociation of cargoes are exemplified by the study of mouse mast cell proteases 6 and 7, since the former remains effectively bound to SG, while the latter dissociates after release from granules (Ghildyal et al., 1996). Dissociation of mouse mast cell protease 7 is dependent on the change in pH during degranulation from acidic to neutral because of key histidine residues required to bind to SG (Ghildyal et al., 1996). Further, equilibration between these extremes has been observed previously for cytotoxic granule proteins. Specifically, granzymes D and E were retained on a CS-A-modified column at acidic pH, but equilibrated between free and bound states at neutral pH (Masson et al., 1990).

Accumulated evidence suggests that degranulated grA and grB are predominantly SG-bound. Through filter fractionation of hCTL degranulate material, whether induced by anti-CD3 (figure 5-1a,b) or Jurkat targets (Table 5-1), the predominantly detected form of grA or grB was SG-bound. This is consistent with previous observations (Galvin et al., 1999). This suggests that neither the drop in concentration, nor the change in pH destabilize these complexes, and is supported by previous characterization. Specifically with respect to concentration effects, by surface plasmon resonance studies, grB-SG binding profiles have indicated that little grB dissociates from SG upon removal of grB from the supernatant, and that some of the dissociated grB may re-bind (Raja et al., 2002). Thus, concentration will likely have a negligible effect on the stability of granzyme-SG complexes. To detail studies of pH effects, grA binding to SG or CS-A is comparable whether at pH 5.5 or 7.4 (Masson et al., 1990). Also, the dissociation

constants of grB-SG at pH 5.5 and 7.4 are relatively comparable (Raja et al., 2002). This is further strengthened by the observation that both grA-SG and grB-SG complexes were stable in the degranulate environment, since neither dissociated over time (figure 5-2 a,b). Thus, change in pH is not a destabilizing factor for grA-SG or grB-SG complexes.

In a seeming contradiction to the evidence that granzyme-SG complexes are not destabilized upon neutralization, when grB-SG complexes were equilibrated at pH 5.5 and 7.0, a smaller pool of grB-SG was detected at neutral than at acidic pH (figure 5-2d). Notably, this difference was not observed with grA (figure 5-2c). The observed pH-dependent difference for grB-SG complexes likely reflects a slight shift in the equilibration point, and the detected grB-SG complexes are not in fact unstable. An equilibrium shift is supported by previous quantification of grB-binding sites on SG (Raja et al., 2002); at pH 5.4 and 7.4, SG is saturated by binding 51 and 32 grB molecules, respectively. Similarly, when grB was complexed to CS-A chains *in vitro*, 85% of grB could be complexed at pH 5.5, but only 63% at pH 7.4 (Galvin et al., 1999). Though conformational change of SG between pH 5.5 and 7.4 was a suggested explanation for these observations (Raja et al., 2002), this seems unlikely. The reasoning is that free CS-A chains would not be subjected to the same conformational constraints as SG, yet grB binding by both CS-A and SG was pH dependent. Rather, it seems more likely that grB packing density would be affected by pH-dependent charge conversion on the surface of grB.

Though it is intriguing to consider the purpose of a small free grB pool that is the result of pH-dependent dissociation, it is unknown at this time whether this would be a physiological factor during granule-mediated killing. First, the rate of this dissociation

event is unknown, thus it cannot be compared to the minutes it can take to deliver grB from the cytotoxic granule to the cytoplasm of the target cell (Sanderson, 1976; Rothstein et al., 1978). Secondly, grB-SG complexes from isolated granules have been characterized with respect to packing density, and only a fraction are packed to saturation (Raja et al., 2002). Thus it is likely that upon degranulation, grB would be expelled only from this small pool of grB-SG complexes.

Up to this point, the data suggest a model that during granule-mediated killing, grA and grB interact with the target cell predominantly in SG-complexed forms. It has been found that grB-SG is specifically directed to the CI-MPR (chapter 4) and a dynamin-dependent pathway (chapter 3) to induce pfn-assisted apoptosis, thus this is likely the physiological pathway. A similar uptake model for grA is a reasonable prediction, for two reasons. First, grA possesses a M6P moiety (Burkhardt et al., 1989), which could mediate binding to the CI-MPR at the cell surface, as has been observed for grB (Motyka et al., 2000). Indeed, grA has been found to bind to the CI-MPR in M6P-dependent manner (figure 5-4a,c,d). Secondly, grA maintains a stable complex with SG after degranulation (figure 5-2a), thus SG might have a refining role in directing grA trafficking, as has been observed for grB (chapters 3 and 4).

One concern that surrounded CI-MPR and granzyme-SG binding was whether the complex could remain intact while binding to the CI-MPR, or whether SG would dissociate after ushering granzyme to the receptor. Data have been presented to suggest that in fact SG-granzyme-CI-MPR form a stable interaction (figure 5-4f). When degranulate material was incubated with CI-MPR, a M6P-dependent physical link was detected between SG and CI-MPR. Since grB and grA also bound to the CI-MPR in a

M6P-dependent manner, the most likely interpretation is that granzymes are the molecular bridge between SG and the CI-MPR, though other M6P-modified granule molecules could function similarly. Notably, binding of SG to the cell surface has received previous indirect support through ultrastructural studies. Specifically, associations have been detected between the target cell membrane and dense cytotoxic granule cores (Peters et al., 1989), which contain SG along with other cytotoxic molecules (Burkhardt et al., 1990; Peters et al., 1991).

The proposal of a SG-granzyme-CI-MPR interaction has several important implications. To begin, this disproves the proposal that SG might prevent binding of grB or grA to the CI-MPR, whether by masking the M6P or by providing steric hindrance (Metkar et al., 2002). Next, this provides further indirect support for CI-MPR-mediated uptake of grA. Finally, this also raises the possibility that a single receptor-granzyme interaction facilitates the internalization of a whole SG complex. If this indeed occurs, then other types of cytotoxic granule factors may be internalized due to a physical link with granzyme, which is SG-mediated. Notably, however, grA internalization likely will not co-internalize grB, or vice versa, since no physical link could be detected between these molecules, despite the fact that each remained bound to SG (figure 5-5). It should be noted that, as unexpected as this result might be, this is consistent with previous data. Granzyme release was monitored from a stimulated human CTL clone, and showed that grA concentrations increased linearly from the starting time, while significant increases in grB concentration were only detectable after the third hour (Spaeny-Dekking et al., 1998). Since grA and grB were not released simultaneously, this would suggest that they are targeted to separate granules. Conceivably, this could be achieved by pathways that

target granzymes to distinct granules, or by distinct sub-populations of CTL that preferentially express one granzyme or the other.

In contrast to grA and grB, which form complexes with SG that are stable after degranulation, pfn does not form a stable interaction with SG in the extracellular environment. This is supported by detection of a labile, high-molecular weight pfn complex (figure 5-3), and is consistent with previous findings that a pH shift from 5.5 to 7.4 destabilizes pfn-SG binding (Masson et al., 1990). However, detection of this complex in degranulate in the first place (figure 5-3, time 0 h) is also consistent with the ability to form a significant, albeit small pool of pfn-SG complexes *in vitro* (Metkar et al., 2002). Though the relative function and importance of free versus SG-bound pfn are unknown, preferential dissociation of pfn from SG in the extracellular environment might serve two purposes. First, pfn might only interact with the target cell and mediate granzyme delivery when pfn is in its free form. Secondly, because grB and pfn can be physically linked by binding to SG (Metkar et al., 2002; Raja et al., 2002), leakage and diffusion of such a complex to peripheral cells would be lethal due to co-delivery of grB and pfn. In contrast, dissociation of pfn causes a physical separation from grB, and diffusion of the two entities would minimize the likelihood that pfn and grB would reunite to act on innocent bystanders.

In conclusion, a picture of granule-mediated killing is emerging throughout which SG functions are integrally woven. The proposed model suggests that stable grA-SG and grB-SG complexes are secreted during granule-mediated killing. The SG-bound grA or grB associate with the CI-MPR to mediate endocytosis of the whole SG complex. In contrast, the secreted pfn dissociates from the complex to interact with membranes, and

mediate, by an unknown mechanism, the endosomal release of the granzyme-SG complexes. Thus, though the granzymes and pfn are the primary apoptotic effectors of granule-mediated killing, a key player is SG, for which the specific functions and nuances are only beginning to be uncovered.

Chapter 6

Summary and Future Directions

6.1. Summary

During granule mediated killing by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, an array of molecules are released. These molecules include a proteoglycan, serglycin (SG), and cytotoxic effectors, including the serine proteases granzyme A (grA) and granzyme B (grB), and the pore-forming molecule, perforin (pfn). In this thesis, the models that have developed predominantly surround the activities of SG. First, a hypothesis was tested that SG would affect grB uptake pathways. Then, having found truth in that model, the stability and nature of the grB-SG and grA-SG complexes were characterized.

Regarding grB uptake, the first hypothesis was that grB entered target cells by a dynamin-dependent endocytic event (chapter 3). When dynamin-dependent and, thus, clathrin-mediated endocytosis were blocked by overexpression of dominant-negative dynamin, grB internalization was only partly blocked. This suggested that grB could enter cells by both dynamin-dependent and -independent routes. Further, dominant-negative dynamin only partially blocked grB and pfn-mediated apoptosis, therefore entry of grB by either pathway was sufficient to induce apoptosis. In contrast, when grB was complexed to SG and then co-delivered with pfn to induce apoptosis, now dominant-negative dynamin was effectively inhibitory. This indicated that delivery of SG-bound grB was critically dependent on dynamin. This finding received further support through CTL-mediated killing assays.

The second hypothesis tested was that grB endocytosis is mediated by the cation-dependent mannose 6-phosphate receptor (CI-MPR; chapter 4). Target killing by isolated

granules could be blocked by the competitive inhibitor, soluble mannose 6-phosphate (M6P). In contrast, killing by grB and pfn was minimally affected by the inhibitor. One potentially distinguishing factor was that grB in isolated granules would be complexed to SG. To test this, grB was complexed *in vitro* to the glycosaminoglycans (GAGs) heparin or heparan sulfate, then used to kill targets in the presence of pfn. Under these conditions, M6P was an effective inhibitor. As a further exploration, grB-SG complexes were obtained in CTL degranulate material, and were used with streptolysin O to kill target cells. Once again, cell death was completely sensitive to M6P inhibition. Together these data suggested that complexing grB to SG preferentially directs grB to a CI-MPR-mediated uptake pathway.

The combined results of the two studies suggested that SG has a specificity-inducing effect on grB-cell surface interactions. And given the critical dependence of grB-SG and pfn-mediated killing on both dynamin and the CI-MPR, it is likely that these are factors along an identical pathway. A likely mechanism by which SG effects its refining role is to interact with the positive charges on grB, so that those will be inaccessible to mediate non-specific interactions between grB and negatively-charged cell surface molecules, such as GAGs or phospholipid headgroups.

Finally, given the different behaviours of free grB and the grB-SG complex, the properties and physiological relevance of granzyme-SG complexes were explored (chapter 5). The major form of granule-released granzymes was SG-bound, and these granzyme-SG complexes were highly stable in the extracellular milieu, suggesting that these complexes are highly relevant to the physiological model. This complex was also stable when granzymes bound to the CI-MPR, suggesting that the whole complex might

be internalized during a single endocytosis event. Despite this prediction, grA and grB will likely not be co-internalized, because these molecules could not be co-precipitated from degranulate material, despite the fact that both bound stably to SG.

Altogether, the data imply a model that during granule-mediated killing, endocytosis of grA-SG or grB-SG into the target cell will be mediated by the CI-MPR (figure 6-1). The role of pfn is to mediate the release of the complex from the vesicle, and thereby expose the granzymes to their cytoplasmic substrates to activate the apoptotic program.

6.2. Future Directions

6.2.1. Serglycin and Implications for Granzyme Uptake

To begin, a model has been formulated that predicts grB-SG and pfn-mediated killing is CI-MPR dependent. Notably the data supporting this model depended on killing by GAG-bound grB and pfn (figure 4-3), or by SG-bound grB and streptolysin O (figure 4-4). Thus the model has not been tested in its truest form, namely, with SG-bound grB and pfn. This question readily could be tested.

Along the same lines, a similar uptake model can be proposed for grA. This is a reasonable proposition because grA possesses a M6P modification (Burkhardt et al., 1989) that binds to the CI-MPR (figure 5-4), and because degranulated grA is stably bound to SG (Masson et al., 1990; and figure 5-2a). Further, the grA-SG complex may bind as a stable unit to the CI-MPR (figure 5-4). Even so, conclusive evidence is lacking for this model.

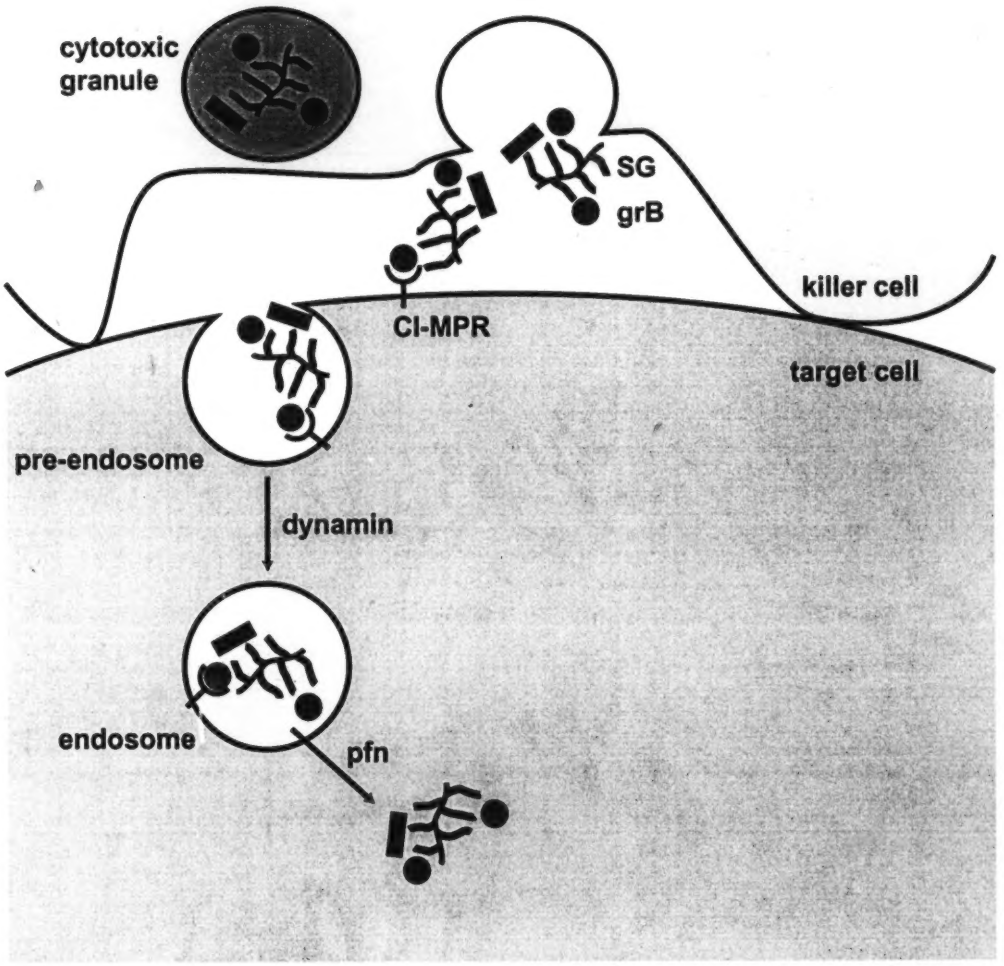

Figure 6-1. Physiological model of granzyme B uptake.
GrB forms a stable complex with SG, both in the cytotoxic granule and the intercellular space. The SG-complexed grB specifically binds to the CI-MPR, and enters a pre-endosome. In a dynamin-dependent event, the endosome pinches-off from the plasma membrane. Pfn then causes release of the grB-SG complex from the endosome into the cytoplasm, where apoptosis is induced. Notably, other SG-complexed materials may be trafficked through the same pathway as tag-alongs.

One curious observation specific to grB was that SG and grB associated more efficiently at acidic pH than at neutral pH (Raja et al., 2002; and figure 5-2 d). This implies that due to a change in environmental pH, some degranulated grB will dissociate from SG. Theoretically, the free grB pool might have an important mechanistic impact for grB uptake during granule-mediated killing, since the uptake pathway of free grB can be CI-MPR-independent (Trapani et al., 2003). However, it is still unknown whether the formation of a free-grB pool would be physiologically relevant during granule-mediated killing. Firstly, the rate of grB-SG dissociation is unknown, and may not occur in the time-frame in which of the death message is delivered from the cytotoxic granule to the cytoplasm of the target cell, an event that sometimes requires only minutes (Zagury et al., 1975; Rothstein et al., 1978). Secondly, the size of the free-grB pool has not been satisfactorily quantified, and may be of insufficient size to have significant consequence on grB endocytosis events. Thus the relevance of a free-grB pool in target cell uptake cannot even be speculated until these questions have been addressed.

Despite an emphasis on the CI-MPR-mediated pathway as a physiological mechanism for grB uptake, it is clear that in the absence of SG, grB and pfn-mediated killing does not require this specificity (Trapani et al., 2003; Kurschus et al., 2005; and figures 4-2 and 4-4). These different modes of grB internalization might be exploited if grB were used for therapeutic purposes. For example, if grB were utilized as an anti-cancer agent in a tumour that had down-regulated the surface expression of the CI-MPR, delivery of grB in the absence of SG could take advantage of the non-specific uptake pathways. Alternatively, if grB were tagged to mediate uptake by another receptor, its delivery in complex with SG could enhance the specificity of internalization. It must be

noted, however, that SG has been found to stimulate CTL activity (Toyama-Sorimachi et al., 1995), and to affect adhesion of lymphoid cells (Toyama-Sorimachi et al., 1997). Thus delivery of SG for therapeutic purposes has complex implications that first require further study.

6.2.2. Serglycin and Implications for Granule-Mediated Killing

Given the inherent stability of granzyme-SG complexes, even when bound to the CI-MPR (figure 5-4f), it is likely that the impact of SG on granzymes will not be restricted to uptake mechanisms. In order to assess this, first it should be determined conclusively that intact SG complexes are internalized by endocytosis. This is a relevant concern since the SG-granzyme-CI-MPR interaction was detected under isolated conditions, whereas at the cell surface, factors might induce subsequent dissociation of SG from receptor-bound granzyme. One approach might be to metabolically label killer cells with [^{35}S]sulfate to tag SG. Next, degranulate material could be collected, then applied to target cells. Uptake of SG into the cells would then be monitored by accumulation of the radiolabel. As a crude control for granzyme uptake in this system, granzymes could be tracked if they were metabolically labeled with a source of ^{32}P. Once the results of this assay have been determined, then further implications may be assessed.

Assuming that granzyme-SG complexes remain as intact units during all of granzyme-induced apoptosis, it is predictable, that SG might impact enzymatic activity of the granzyme. Two possible mechanisms by which this could occur is by affecting interactions both with physiological inhibitors and with substrates. Regarding

interactions with inhibitors, evidence suggests that SG-bound grA is less susceptible to protease inhibitors in plasma, such as antithrombin III and α-2-macroglobulin (Spaeny-Dekking et al., 2000). Thus, a grA-SG complex might have reduced susceptibility to any unidentified cytoplasmic grA inhibitors. Along the same line, complexing grB to SG might reduce interactions with PI-9, a cytoplasmic inhibitor which may provide protection to some immune cells, immune privileged sites, and tumours (reviewed by Trapani and Sutton (2003)). With respect to granzyme substrate interactions, it should be noted that grB-SG-mediated cleavage of caspase 3 and caspase 7 has been considered previously (Metkar et al., 2002). The relative kinetics of substrate cleavage by grB-SG were dependent on the conditions of the assay. When the caspases were cleaved in a neutral saline solution, then grB-SG was less efficient than free grB (Metkar et al., 2002). In contrast, when cleavage was performed in cell lysates, then grB-SG was equally as effective as free grB (Metkar et al., 2002). It is tempting to speculate that under physiological conditions, grB activity likely will not be affected by binding to SG. However, it is also evident that this is a complex issue that requires careful study.

On the topic of additional SG function in granule-mediated killing, the purpose of pfn-SG has largely been ignored. Indeed, pfn-SG complexes can be generated *in vitro* under extracellular conditions (Metkar et al., 2002). Also, a labile pool of pfn-SG complexes may have been detected in degranulate material (figure 5-3). It has even been found that pfn-SG has reduced lytic activity, though is not affected in its ability to deliver grB (Metkar et al., 2002). However, this still does not reveal the function pfn-SG, or whether it even has one. Since the activities of free pfn are also poorly understood, perhaps comparative study of free and SG-bound pfn will help reveal the secrets of each.

It is predictable that cytotoxic granule molecules other than grA, grB, or pfn might also be influenced by SG after degranulation. Interestingly, though cytotoxic granules contain both granule-specific and lysosomal factors, these likely will not be affected in a similar manner. Ultrastructural studies generally indicate a segregation of the cytotoxic proteins, such as granzymes and perforin, from the lysosomal proteins, such as cathepsin D and lamp-1 (Peters et al., 1989; Burkhardt et al., 1990; Peters et al., 1991). Specifically, the former concentrate in the dense core, along with SG (Burkhardt et al., 1990), while the latter distribute to a multivesicular cortex just under the delimiting membrane. Thus, good candidates to interact with SG are the cytotoxic proteins, including orphan granzymes (Grossman et al., 2003) and granulysin (Clayberger and Krensky, 2003). In contrast, the lysosome-type proteins are less likely to have this interaction.

A further aspect to loading SG with cytotoxic granule molecules is the mixing of cargo in one SG complex. Evidence has been presented to suggest that grA and grB are segregated to distinct pools of SG (figure 5-5), thus it would be interesting to consider mechanisms by which this occurs. Reasonable hypotheses include trafficking pathways to distinct granules, or preferential expression of either granzyme by killer cell sub-populations. Though grA and grB were found to be segregated, it is unlikely that all cytotoxic effectors are segregated to distinct pools of SG. Thus, one consideration is which molecules are physically linked by binding to SG, and therefore might be co-delivered by a single receptor-mediated endocytosis event. Perhaps co-delivery of specific effectors will help coordinate activation of the apoptotic pathway through co-targeting of effectors to substrates.

While the focus of this study has surrounded the impact of SG on granule-mediated killing, it is still unknown whether SG is in fact essential for granule-mediated killing. From mice defective in SG expression, evidence suggests that SG is critically involved in the formation of secretory granules in mast cells (Humphries et al., 1999; Forsberg et al., 1999; Åbrink et al., 2004). Though this was not explored in the deletion studies, likely the same would be true in CTL and NK cells. This issue has been examined through the use of a GAG acceptor molecule, PNP-xyloside, to prevent glycosylation of the SG core peptide (Masson et al., 1990). Strikingly, these conditions had no effect on target cell lysis induced by pre-treated CTL. However, after PNP-xyloside treatment, free GAG chains were detected in the granules, and these might have compensated for the absence of intact SG proteoglycans. A more effective approach to study these questions would be analysis of killer cells from the SG knockout mice (Åbrink et al., 2004). It would then be possible to explore the specific fate of the granule proteins in the absence of SG, as well as the issue of killer cell cytotoxicity. And if granzyme-mediated cytotoxicity were retained in the absence of SG and GAGs, it would also be interesting to test whether grB-mediated killing mechanisms are now CI-MPR-independent.

In conclusion, SG activities have been detected in the extracellular space, particularly in affecting grB endocytosis. This raises the possibility that other cytotoxic granule factors are similarly influenced by complexing to SG. This research also raises the relative awareness of SG as a molecule with function, and may inspire further exploration of its activities in both granzyme uptake and granule-mediated killing.

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